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Azorean macroalgae (*Petalonia binghamiae*, *Halopteris scoparia* and *Osmundea pinnatifida*) bioprospection: A study of composition and bioactivity

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I. Abstract

The Azores archipelago (in mid-Atlantic) has a substantial variety of seaweed species. In particular, the brown macroalgae species *Petalonia binghamiae* and *Halopteris scoparia*, and the red macroalga *Osmundea pinnatifida* are still undervalued and not thoroughly studied. As a contribution to the bioprospection of these three species, proximal composition, lipid composition, phenolic content and relevant bioactivities were determined. Moisture was low in all samples since they were previously sun-dried. Protein content ranged from 10.3-14.4% dw. Mineral content was relatively high for all species, particularly for *H. scoparia* (51.9% dw). Total carbohydrate content ranged from 32.5-48.3% dw, being the highest value attained for *P. binghamiae*. The three seaweed species had a low fat content with predominance of non-polar lipids, mainly free fatty acids (FFA) and triacylglycerols (TAG). Their fatty acid (FA) profile was characterized by abundance of saturated FA (SFA) in the case of *O. pinnatifida* and a similar weight of SFA and polyunsaturated FA (PUFA) in the other two species. Monounsaturated FA' (MUFA) share was relatively small in all species. Within PUFA, ω3 PUFA were the most abundant in *O. pinnatifida* and *P. binghamiae*, thus yielding ω3/ω6 ratios clearly higher than one. The palmitic acid (16:0) content was very high and the myristic acid (14:0) content was substantial in all species. Oleic acid (18:1 ω9) level was also high, particularly, in *P. binghamiae*, reaching 16.95 ± 0.05% of total FAs. Linoleic acid (18:2 ω6) content was in the 7-13% range with exception of *O. pinnatifida*, whose linoleic acid content was low, 1.34 ± 0.01%. Arachidonic acid (20:4 ω6) level was similar or higher than linoleic acid level in all studied species. Regarding ω3 PUFA, eicosapentaenoic acid (EPA, 20:5 ω3) was the most significant (10-14 %) with exception of *H. scoparia*, which was richer in stearidonic acid (SDA, 18:4 ω3) and α-linolenic acid (ALA, 18:3 ω3) than in EPA, 7.60 ± 0.03% and 6.73 ± 0.03% *vs* 5.63 ± 0.03%, respectively. The highest EPA concentration was found in *P. binghamiae* (176 ± 4 mg/100 g dw). Concerning other bioactives, while β-glucans (laminarin) were only detected at trace levels, polyphenols were present at non-negligible levels, reaching 140-220mg/100g dw. The 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Ion Reducing Antioxidant Power (FRAP), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) methods showed some antioxidant activity, especially in aqueous extracts of *H. scoparia* and *P. binghamiae* assessed by ABTS. Ethanolic extracts of *H. scoparia* and aqueous extracts of *P. binghamiae* showed inhibitory capacity of cyclooxygenase-2 (COX-2), of 40 and 79%, thus indicating some anti-inflammatory activity.

Keywords: Rhodophyta; Phaeophyceae; lipid profile; antioxidant activity; anti-inflammatory activity.

II. Resumo

As macroalgas habitam os oceanos há mais de 2 biliões de anos e há registos da sua inclusão na dieta de muitos povos asiáticos desde o século XVII. Estes organismos multicelulares têm vindo a ser alvo da atenção da comunidade científica devido às suas propriedades e possíveis aplicações em diversas áreas da indústria, sendo usados como alimentos, fertilizantes, cosméticos e até medicamentos. Diversos compostos extraídos de macroalgas têm vindo a revelar propriedades e bioatividades de extremo interesse. Por exemplo, o ácido eicosapentaenóico (EPA) é o ácido gordo predominante na maioria das espécies e exibe atividades antioxidante e anti-inflamatória muito significativas. Outros compostos, como o fucosterol e alguns péptidos podem ajudar na prevenção do aumento do colesterol e da pressão arterial, bem como ter uma ação antiviral e antimicrobiana. Os pigmentos são também de extrema importância, sobretudo a fucoxantina que é muito abundante nas algas castanhas, uma vez que possui ações anti-carcinogénicas e pode ajudar no controlo da obesidade e da diabetes. As macroalgas são também conhecidas pelo seu alto teor em minerais, sendo por isso uma excelente fonte de micronutrientes, tais como iodo, cálcio, magnésio e potássio. No entanto, visto que as algas são bioacumuladores e a poluição do ambiente marinho tem vindo a crescer significativamente, alguns destes minerais podem ser acumulados em quantidades acima do desejado passando a ter uma ação tóxica, como acontece por vezes no caso do cobre, do arsénio e do chumbo.

Muitos estudos científicos têm vindo a ser realizados ao longo dos últimos anos, mas as macroalgas são organismos com tanta diversidade inter e intraespecífica que ainda existem muitas espécies cuja composição e propriedades são praticamente desconhecidas. Em Portugal, existe também um interesse crescente por estes organismos, especialmente quando se tratam de espécies que já possuem um valor comercial. Este é o caso das espécies *Halopteris scoparia, Petalonia binghamiae* e *Osmundea pinnatifida*, que já são regularmente recolhidas e comercializadas nos Açores. *Osmundea pinnatifida* é uma alga vermelha (Rhodophyta) comestível, conhecida por ter um sabor picante. Em algumas ilhas dos Açores é consumida em *pickle* ou usada seca como especiaria. *Petalonia binghamiae* e *Halopteris scoparia* são algas castanhas e pertencem à classe Phaeophyceae. A primeira trata-se também de uma alga comestível, sendo abundantemente consumida em países como a China, o Japão e a Coreia. *H. scoparia* é a única das três algas incluídas neste estudo que é considerada não-comestível, mas é usada em vários países na indústria farmacêutica e cosmética devido às suas propriedades antimicrobianas.

Este trabalho teve como objetivo efetuar um estudo de bioprospecção da biomassa destas três espécies de macroalga tal como é atualmente tratada e consumida nos Açores (seca ao sol), visto que estas algas são ainda pouco conhecidas e provavelmente subvalorizadas. Para tal, em primeiro lugar foi feita a análise da composição proximal de cada uma destas espécies. Os valores de humidade obtidos foram baixos, tal como era esperado visto que as algas foram previamente secas. Os valores de cinza (minerais) foram relativamente elevados em todas elas, sendo o valor mais baixo registado para a espécie *P. binghamiae* (27,9% do peso seco) e o valor mais alto para a *H. scoparia* (51,9% do peso seco). O conteúdo proteico, calculado pelo método de Dumas, variou entre 10,3-14,4% do peso seco. O conteúdo em hidratos de carbono foi elevado para as três espécies, variando entre 32,5-48,3% do peso seco. Todas as algas revelaram ter um conteúdo lípido baixo, apesar de a espécie *P. binghamiae* se ter destacado com uma percentagem de 4,5% do peso seco. Dois métodos de extração de gordura foram testados, tendo o método de Folch sido o que revelou uma extração mais eficaz. Seguidamente, a distribuição das classes lipídicas de maior interesse foi estudada com recurso a cromatografia de camada fina (TLC). Verificou-se, para as três espécies de macroalga estudadas, a predominância de triacilgliceróis (TAG) e ácidos gordos livres (FFA). A quantificação do conteúdo em fosfolípidos e glicolípidos não foi possível, muito possivelmente devido ao facto de as amostras terem sido secas ao sol e estes serem compostos que se degradam facilmente nestas condições. O perfil de ácidos gordos foi caracterizado pela abundancia de ácidos gordos saturados (SFA)

na espécie *Osmundea pinnatifida* e por uma proporção equilibrada entre SFA e ácidos gordos polinsaturados (PUFA) nas outras duas espécies. Dentro da classe PUFA, os ω3 PUFA são os mais abundantes nas espécies *O. pinnatifida* e *P. binghamiae*. O teor em ácido palmítico (16:0) e ácido mirístico (14:0) foi elevado em todas as espécies. O teor em ácido eicosapentaenóico (EPA) foi também significativo, exceto na espécie *H. scoparia* que revelou ser mais rica em ácido estearidónico (SDA, 18: 4 ω3) e ácido α-linoleico. (ALA, 18: 3 ω3). Outros dois compostos bioativos de interesse foram quantificados para as três espécies em estudo: β-glucanos (laminarina) e polifenóis. O conteúdo em β-glucanos detetado foi muito baixo para todas as espécies, mas os compostos fenólicos foram quantificados em níveis bastante consideráveis, atingindo valores de 140 a 220mg / 100g de peso seco. Por fim, duas bioatividades foram testadas para extratos aquosos e etanólicos das três macroalgas em estudo. A atividade antioxidante foi testada com recurso a três métodos diferentes: o método do 2,2-difenil-1-picrilhidrazil (DPPH), o método do Potencial Antioxidante Redutor do Ferro (FRAP) e o método do ácido 2,2'-azino-bis (3-etilbenzotiazolino-6-sulfónico) (ABTS). Foi demonstrada a existência de atividade antioxidante considerável para as três macroalgas, especialmente em extratos aquosos de *H. scoparia* e *P. binghamiae*. A atividade anti-inflamatória foi testada através do método da inibição da ciclooxigenase-2 (COX-2), e apenas o extrato aquoso de *P. binghamiae* e o extrato etanólico de *H. scoparia* demonstraram capacidade inibitória, mas com valores bastante consideráveis (40% e 79%, respetivamente).

No geral, os resultados obtidos indicam que estas espécies de macroalga têm um enorme potencial e que devem ser melhor exploradas no futuro. Mais especificamente, o alto teor em minerais poderá ser de extrema importância sobretudo no caso da *H. scoparia* que apesar de não ser considerada uma alga comestível poderá vir a ser usada em processos de biorremediação ou para controlo de qualidade dos ambientes marinhos. O conteúdo em polissacáridos revelou-se também promissor, visto que embora a grande maioria dos hidratos de carbono nas algas seja fibra dietética e esta não seja absorvida pelo organismo humano, a sua presença no trato intestinal (especialmente no intestino grosso) contribui muito positivamente para a digestão, o que indica que as espécies estudadas poderão ser usadas como pré-bióticos. A fração lipídica, embora não muito abundante, revelou também resultados muito interessantes sobretudo para a *P. binghamiae*, na qual foi doseada uma percentagem de gordura total considerável e uma prevalência de ácidos gordos ω3, mais concretamente EPA, que é um componente estrutural essencial para as membranas celulares e poderá ser usado como antioxidante ou no controlo de altos níveis de colesterol. O conteúdo fenólico foi significativo para as três espécies, sugerindo a existência de atividade antioxidante, a qual foi posteriormente confirmada, daqui resultando a possibilidade destas algas virem a ser usadas para fins terapêuticos ou cosméticos. A atividade anti-inflamatória presente em duas das algas estudadas reforça a ideia de que estas espécies poderão vir a ter diversas aplicações em diversas áreas da indústria.

Por fim, é importante referir que o processamento efetuado às algas nos Açores (secagem ao sol) poderá não ser o mais indicado, sobretudo quando se trata de compostos que se degradam facilmente, como os glicolípidos. Seria, portanto, interessante realizar um estudo com estas mesmas espécies mas em matéria fresca, de forma a obter um perfil comparativo dos diferentes componentes e das bioatividades testadas. O desenvolvimento de novos métodos de extração é também de extrema importância, sobretudo no caso dos hidratos de carbono que são geralmente estimados por diferença em todos os estudos. A determinação do perfil de minerais é também de extrema importância, tal como a quantificação de outros compostos bioativos como a fucoxantina e os fucanos. Numa fase posterior, a bioacessibilidade de todos estes compostos deverá ser estudada de modo a obter uma perspetiva real da sua disponibilidade para o organismo humano quando ingeridos, uma vez que a matéria vegetal nem sempre é totalmente digerida e absorvida.

Palavras-chave: Rhodophyta; Phaeophyceae; perfil de lípidos; atividade antioxidante; atividade anti-inflamatória.

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VI. List of abbreviations

AA – Arachidonic acid
ABTS - 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ALA - α-linolenic acid
CH – Cholesterol
COX-2 - Cyclooxygenase-2
DAG – Diacylglycerol
DGDG – Digalactosyldiacylglycerol
DHA – Docosahexaenoic acid
DPPH - 2,2-diphenyl-1-picrylhydrazyl
ELISA – Enzyme-linked immunosorbent assay
EPA - Eicosapentaenoic acid
FA – Fatty acid
FAME - Fatty acid methyl esters
FFA – Free fatty acids
FRAP - Ferric Ion Reducing Antioxidant Power
GA – Gallic acid
GAE – Gallic acid equivalents
HPTLC – High performance thin layer chromatography
IL – Interleukin
MAG – Monoacylglycerol
MGDG – Monogalactosyl diacylglycerol
MUFA – Monounsaturated fatty acid
NO – Nitric oxide
PBS - Phosphate buffered saline
PC – Phosphatidylcholine
PE – Phophatidylethanolamine
PGE2 - Prostaglandin E2
PGs – Prostaglandins
PSY – Psychosine
PUFA – Polyunsaturated fatty acid
SDA - Stearidonic acid
SFA – Saturated fatty acid
SPH – Sphingomyelin
SQDG - Sulfoquinovosyl diacylglycerol
TAG – Triacylglycerol
TLC – Thin layer chromatography
TNF – Tumor necrosis factor
TPTZ - 2,4,6-tripyridyl-s-triazine

1. Introduction

1.1. Marine Macroalgae

Marine macroalgae are multi-cellular photosynthetic organisms which live in marine locations, producing oxygen and organic compounds that serve as food for other living beings. So, they are considered primary producers and are fundamental to food chains of all aquatic ecosystems (Pereira and Correia 2015). Some species can be several meters long and exhibit a very complex tissue organization. Macroalgae (or seaweeds) can show a wide variety of colours depending on the combination of different photosynthetic pigments in their cells. The taxonomy of these organisms is in part based on the colour they exhibit. Green seaweeds belong to the phylum Chlorophyta and have pigments such as chlorophylls a, b and carotenoids. Seaweeds that exhibit a red coloration belong to the phylum Rhodophyta and their main pigments are chlorophyll, carotenoids and phycobilins. Seaweeds whose predominant pigments are carotenoids, specially fucoxanthin, and have a brownish colour belong to the phylum Heterokontophyta (or Ochrophyta) and are grouped in the class Phaeophyceae (Pereira 2016).

1.2. Seaweed properties

1.2.1. Lipids and fatty acids

The total lipid content in macroalgae is generally in the range of 1-6% dry weight, although in some brown seaweeds can go up to almost 20% (Miyashita et al., 2013; Fleurence and Levine, 2016). Most of the lipid fraction in macroalgae is composed by glycolipids, being phospholipids the second most abundant type of lipid, followed by triacylglycerols and sterols (Holdt and Kraan, 2011; Miyashita et al., 2013). Though in most seaweeds the lipid fraction is small, it contains several important bioactive components such as fucoxanthin, polyphenols and polyunsaturated fatty acids (PUFA) (Airanthi et al., 2012; Eko Susanto et al., 2016).

Glycolipids and Phospholipids

Glycolipids are polar carbohydrate-attached lipids and play a major role in cellular recognition because of their association with cell membranes. They also act as energy providers, ensuring processes such as photosynthesis (Miyashita et al., 2013). In macroalgae, the glycolipid content is usually between 20% and 50% of total glycolipids and is usually composed mainly by monogalactosyl diacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyl diacylglycerol (SQDG) (Dembitsky et al. 1991).

Phospholipids are also polar compounds that consist of fatty acids and a phosphate attached to a glycerol or the amino alcohol sphingosine. Phosphatidic acid, phosphatidylcholine (PC), phophatidylethanolamine (PE), phosphatidylinositol, phosphatidylserine and sphingomyelin (SPH) are the ones worth mentioning for their abundancy and biological importance. Besides the major role phospholipids play in cell structure and transport, they also contribute to muscle control, memory storage, digestion and the nervous system. In seaweed the predominant phospholipid is usually PC (Holdt and Kraan, 2011).

Sterols

Cholesterol, the most familiar type of sterol in animals, is essential to cellular function because it affects the fluidity of the cell membrane. In addition, it is involved in cell signalling and is a precursor to fat-soluble vitamins and steroid hormones (Hold and Kraan, 2011).

In seaweeds cholesterol is not the only important sterol and different types of seaweed can have different sterol contents. While green seaweed species are richer in cholesterol and 28-isofucocholesterol, red and brown seaweed contain mostly fucosterol (24-ethylidene cholesterol), this being around 95% of the total sterol content in some genera like *Laminaria* and *Palmaria* (Holdt and Kraan, 2011). Fucosterol, when isolated from seaweed, can have many applications in the nutraceutical and pharmaceutical fields, since it exhibits several biological activities of interest, including antidiabetic, antioxidant, hepatoprotective, antifungal, antihistaminic, anticholinergic, antiadipogenic, antiphotodamaging, anti-osteoporotic, blood cholesterol reducing and blood vessel thrombosis preventive (Abdul et al., 2016).

Fatty acids

Polyunsaturated fatty acids are essential nutrients which cannot be efficiently synthesized by mammals and therefore must be part of their diet. Omega-3 (ω3) PUFA and omega-6 (ω6) PUFA are of particular interest for its use in functional food. Eicosapentaenoic acid (EPA; 20:5 ω3) and docosahexaenoic acid (DHA; 22:6 ω3) are the two important fatty acids provided by marine lipids, along with the precursor α-linolenic acid (ALA; 18:3 ω3) and docosapentaenoic acid (22:5 ω3) (Holdt and Kraan, 2011). If these fatty acids are ingested in a balanced and correct way can have a very positive impact on health. DHA has been shown to be present in brain cells and a key element in the development and functioning of the nervous system. Similarly, several studies have shown that EPA exhibits significative antioxidant and anti-inflammatory activities (van Ginneken et al., 2011). In macroalgae the predominant fatty acid is usually EPA (20:5 ω3), which can be 50% of the total fatty acid content. Marine algae are also the only organisms that contain the PUFA 18:4 ω3 (Dawczynski et al. 2007; Murata and Nakazoe 2001). Like other biochemical components, the fatty acid content varies with the season and other environmental factors (Holdt and Kraan, 2011).

1.2.2. Polysaccharides

Polysaccharides are polymers of simple sugars (monosaccharides) connected by glycosidic bonds. Their characteristics allow them to be used in numerous products such as stabilisers, thickeners, emulsifiers and beverages (McHugh 1987; Tseng 2001). Marine macroalgae are very rich in these components, both cell wall structural polysaccharides and storage polysaccharides, being their total concentration up to 76% dry weight in some species. Structural polysaccharides mainly consist of cellulose and hemicellulose and their composition and proportion is species specific. Brown algae are usually rich in fucoidan and laminarin (β-1,3 glucan), green algae contain sulphated galactans and xylans, while in red algae agars and carrageenans are the predominant polysaccharides (Murata and Nakazoe 2001; Kumar et al. 2008). Numerous studies have been conducted in order to understand the functions and applications of algae polysaccharides.

One of the most well-studied polysaccharides are sulfated fucans, that contain the monomer fucose. They can occur in a few red and green algae but are mainly found in the cell walls of brown seaweeds (Pomin and Mourão, 2008). Although the major physiological purposes of fucans in macroalgae are not thoroughly understood, these polysaccharides are of major interest because of their properties and the many human health applications they can have. It has been demonstrated that fucans can act as really powerful anticoagulants, have antiviral and anticancer properties (reducing both growth and diffusion of tumors), stimulate and modulate the immune system and also be good antioxidant and anti-inflammatory agents (Ellouali et al. 1993; Zhuang et al., 1995; Chevolot et al., 1999; Usov et al., 2001; Li et al. 2008; Holdt and Kraan, 2011).

Other important brown algae polysaccharide is laminarin. It has a molecular weight of only approximately 5 kDa and is composed of (1,3)-β-D-glucan and some β-(1,6)-intrachain links. The laminarin content varies seasonally and with the habitat but can reach up to 35% of dry weight in some species. This polysaccharide has been claimed to display strong antioxidant (Kadam et al., 2015), anticoagulant, immunomodulatory (Sweeney et al., 2017), anticancer, and anti-inflammatory activities (Kadam et al., 2014).

Non-digestible polysaccharides are called dietary fiber, which can be very diverse in composition and therefore in their properties and biological effects on animal and human health. This fiber can be classified into two types: insoluble and water-soluble. Even though they are not completely broken down by digestive enzymes, both types of dietary fiber are important to the digestive process, being able to delay gastric emptying (soluble fiber) and easing defecation (insoluble fiber). In marine macroalgae, the insoluble portion is mainly composed by cellulose, mannans and xylan, while the water-soluble fraction is rich in polysaccharides such as agars, laminarin, alginic acid and porphyran (Holdt and Kraan, 2011). Numerous studies demonstrate that seaweeds are an important source of dietary fiber, mainly the soluble fractions, and some edible species can contain up to 60% total fiber on a dry weight basis (Lahaye, 1991). For example, *Undaria sp.* and *Fucus sp.* both have their total dietary fiber content around 50% (Murata and Nakazoe, 2001). In addition to the role that dietary fiber plays directly in the digestive process, some of these compounds are also considered important in preventing constipation, colon cancer, cardiovascular disease and obesity, among others (Stephen & Cummings, 1980).

1.2.3. Proteins

The protein fraction of macroalgae is highly variable. In brown seaweeds is usually small, being the maximal content around 24% dry weight in *Undaria*. Red and green seaweeds show higher contents of protein, reaching up to 44% dry weight in some species like *Porphyra tenera* (*nori*) and *Ulva* spp. The amount of protein also depends on the time of the year and life cycle of each seaweed (Arasaki and Arasaki, 1983).

The structure and biological properties of seaweed proteins are in most cases not well documented, but there are lots of studies about the amino acid composition of several species (Murata and Nakazoe, 2001; Holdt and Kraan, 2011). In general, seaweeds contain all the essential amino acids and are rich in acidic amino acids, aspartic acid and glutamic acid. In *Sargassum sp* and *Fucus sp*, the combined glutamic acid and aspartic acid level accounts for around 40% wet weight of the total amino acid fraction, though some reports show that red macroalgae appear to contain lower aspartic and glutamic acid compared to the other two algal groups (Fleurence 2004; Holdt and Kraan, 2011).

The free amino acid fraction is usually composed of alanine, amino butyric acid, taurine, ornithine, citrulline and hydroxyproline. In edible algae, the proportion of these amino acids can influence taste. For example, the high amounts of alanine, glutamic acid and glycine present in *nori* are responsible for its strong and distinctive flavour (McHugh, 2003).

Some important peptides can also be found in macroalgae. For example, carnosine and glutathione, known for their strong antioxidant properties, are usually present in animal tissues but have also been identified in seaweed (Shiu and Lee, 2005). Numerous scientific studies also show that algal peptides can assist in the prevention and treatment of diseases such as high blood pressure, high cholesterol, AIDS and some types of cancer (Sato et al. 2002; Smit 2004, Suetsuna et al. 2004; Holdt and Kraan, 2011).

Other relevant group of proteins present in seaweeds are carbohydrate-binding proteins, or lectins. These proteins are involved in many biological processes such as recognizing and binding carbohydrates, host-pathogen interactions, cell-cell communication, apoptosis, cancer metastasis and differentiation (Hori et al., 2000). Some marine algal lectins can also show important bioactivities, including antibiotic, anti-inflammatory, cytotoxic and even anti-HIV (Bird et al. 1993; Smit 2004; Mori et al. 2005). Some of these proteins are currently being purified from marine algae to be produced and used in the pharmaceutical industry (Holdt and Kraan, 2011).

1.2.4. Pigments

Algal pigments can be divided in three major groups: chlorophylls, carotenoids and phycobiliproteins (pigmented phycobilins).

Chlorophylls are green lipid-soluble pigments found in all algae, higher plants or cyanobacteria that carry out photosynthesis. Chlorophyll content varies with the type of seaweed and with the time of the year.

Like chlorophylls, carotenoids are also present in all algae but represent photosynthetic pigments in the red, orange or yellow wavelengths. This type of pigment is very important because it acts as an antioxidant by inactivating reactive oxygen species formed by exposure to light and air. Some carotenoids can be found in all types of macroalgae, like β-carotene, but others are more specific. For example, red seaweeds also contain zeaxanthin and brown seaweeds are richer in fucoxanthin. This last pigment is one of the most abundant carotenoids in nature and it can have a positive impact on human health. It has been shown that fucoxanthin can have significant anti-cancer and anti-obesity activities (Miyashita and Hosokawa 2008).

Contrary to chlorophylls and carotenoids, phycobiliproteins are water-soluble and are found on the surface of the thylakoids. These pigments can absorb different wavelengths depending on the combination of phycoerythrobilin (red) and phycocyanobilin (blue) they possess and play an important role in the photosynthetic process of some algae. Currently phycobiliproteins are used as a natural food colourant (Houghton, 1996). However, these pigments can have other interesting applications since they have been shown to have important properties such as antioxidant, anti-inflammatory, neuroprotective and antiviral activities (Holdt and Kraan, 2011).

1.2.5. Essential and toxic elements

Some elements play important roles in numerous biologic processes and are therefore essential for the well-functioning of the human organism. The mineral content (or ash) of marine algae is known to be high, being in some cases close to 100 times higher than the same content in traditional vegetables (Arasaki and Arasaki 1983; Nisizawa 2002). Ash can reach up to 55% dw in some species, meaning seaweeds can potentially have enough micro-elements to fulfill the recommended daily intakes in human nutrition (Kumar et al., 2011).

The most abundant minerals in macroalgae are magnesium, calcium, iron, copper, sodium, potassium, zinc, manganese, cobalt and specially iodine. Species of the genus *Laminaria* are the strongest iodine accumulators among all living systems and have been used in Asia as a dietary iodine supplement to prevent goiter, since this element is essential to produce the thyroid hormones thyroxine and triiodothyronine (Holdt and Kraan 2011). Magnesium is also abundant in most marine algae and it's a crucial element for human health because it works as co-factor for DNA and protein synthesis, oxidative phosphorylation, neuro-muscular excitability, enzyme activity and regulation of parathyroid hormone secretion (Romani 2011). Other elements such as zinc and copper are needed in smaller quantities but are equally essential and its non-sufficient intake can lead to several health problems including thymic atrophy, pregnancy complications and vision loss (Macartain et al., 2007; Pineles et al., 2010, Olivares et al., 2011).

However, because of the increased levels of pollution in the oceans, seaweeds also tend to bioaccumulate elements that may have a negative effect on health such as lead, mercury, arsenic and cadmium. The toxicity of these elements is related to reactions that can damage DNA, leading to increased mutagenicity, teratogenicity, genotoxicity, and carcinogenicity (Afonso, 2009). Lead, per example, contaminates the environment due mostly to industrialization and the use of petrol as fuel and even though its levels are decreasing it is still a concerning toxic element because when ingested in excess can lead to hematopoietic system and central nervous system problems (Goyer and Clarkson, 2001; Afonso, 2009). Inorganic arsenic is another toxic element worth mentioning because it also has a negative effect on the human nervous system. Arsenic concentrations are generally considerably higher in brown seaweed than in red or green (Francesconi and Edmonds 1996) and even though part of it is transformed in non-toxic organic arsenic by the biological system, in some cases the inorganic form can still be present in concerning amounts. For instance, the edible seaweed *Sargassum fusiforme* is known to accumulate large amounts (up to 88 mg/kg dw) of arsenic in its inorganic form. The seaweed's ability to accumulate toxic elements can be dangerous when we consider their direct consumption or the bioaccumulation of such elements in the food chain. It is also possible to take advantage of this accumulation in bioremediation processes in the sea or applied to wastewater, making macroalgae an excellent bioindicator (Holdt and Kraan, 2011).

1.2.6. Antioxidant properties

Antioxidants are substances that protect living tissues against damage caused by free radicals. These radicals are produced as a result of the incomplete reduction of the oxygen molecule during aerobic respiration and are directly related to ageing and diverse diseases (Krishnaiah, Sarbatly, & Nithyanandam, 2011). The human organism can partially inhibit the action of free radicals, but antioxidant compounds must also be ingested to help the regulatory system become more effective.

In order to evaluate the antioxidant activity of a substrate some simple chemical tests have been developed: ABTS, DPPH and FRAP assays. Antioxidant power can be evaluated from the direct inhibition of free radicals by the 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays, or from a redox reaction occurring between the substrate (electron donor) and Fe^{III} ions (electron acceptor) producing Fe^{II} ions by the ferric reducing antioxidant power (FRAP) assay (Martins et al.,2013).

Several studies suggest that seaweed extracts can be considered as a potential source of antioxidants, which might be included in functional food or used in the pharmaceutical industry (Holdt and Kraan 2011). More specifically, brown and red macroalgae are considered rich in antioxidant agents such as pigments (fucoxanthin) and polyphenols. It has even been shown that some seaweed species - *Lobophora variegate* and *Chondria baileyana* - have activities equivalent to those of commercial antioxidants (Zubia et al.,2007). Like many other previously referred properties, the antioxidant levels present in seaweed may be affected by environmental factors such as location, salinity and light exposure (Holdt and Kraan, 2011).

1.2.7. Anti-inflammatory properties

Inflammation of living tissues is a very frequent process that can result in genetic and immunoregulation defects which lead to tissue damage, therefore being linked with many deadly disorders like cancer, neurodegenerative and cardiovascular diseases, diabetes and obesity (Jaswir and Monsur, 2011). In this process, activated inflammatory cells secrete increased amounts of nitric oxide (NO), prostaglandins and cytokines, such as interleukin (IL)-1ß, IL-6, and tumor necrosis factor (TNF). To control the production of these compounds there are important inflammatory mediators, especially nitric oxide synthases (NOS) and prostaglandin E2 (PGE2), being this last one produced from arachidonic acid metabolites by the catalysis of cyclooxygenase-2 (COX-2). However, the overproduction of these mediators can lead to complications, like organ destruction in some inflammatory and autoimmune diseases (Yoon et al., 2009).

There are many artificial anti-inflammatory substances in the market but they are known for also having unwanted side-effects, such as liver damage and heart problems, thus safe biological sources are now being considered as alternatives (Clegg et al., 2006). Plants and seaweeds have been used for centuries to treat inflammatory conditions and recent studies show that some polysaccharides, polyunsaturated fatty acids and carotenoids from macroalgae can really have anti-inflammatory activity and medicinal value (Yoon et al., 2009).

In order to quantify the anti-inflammatory power of such substances there are a number of assays that can be performed, regarding tumor necrosis factor (TNF-α), interleukin (IL 1β, 6, 8), Nitric oxide (NO), inducible Nitric oxide synthase (iNOS), Prostaglandin E (PGE 2 and 3), Cyclooxygenase (COX-2), transcription factor (NF-κB) and leukotrienes (LB 3 and 4) (Jaswir and Monsur, 2011).

1.3. Studied species

1.3.1. *Osmundea pinnatifida*

Osmundea pinnatifida (Hudson), also known as Pepper Dulse (Chapman & Chapman 1980) is a marine seaweed widely distributed in the tidal zone of rocky shores around Europe. It belongs to the Rhodophyta division but, despite being a red seaweed from a taxonomic point of view, it can show a wide range of colors from yellow to almost black (Figure 1.1.). It is also very variable in size depending upon its location on the shore. Higher shore plants are generally smaller and yellow-green in colour, due to exposure to high levels of sunshine while on the lower shore they are reddish-brown. Once dried can be used as a pepper- or curry-flavoured spice and in some islands of Azores is pickled in vinegar with onions and eaten with fried fish (Neto et al., 2005).

Figure 1.1. – Freshly caught exemplar of *Osmundea pinnatifida*, Azores islands, Atlantic Ocean.
SeaExpert, 2017.

1.3.2. *Halopteris scoparia*

Halopteris scoparia (Linnaeus) is a dark brown seaweed (Phaeophyceae) that can be found all the way from Norway to Cape Verde, the Mediterranean and the Black Sea. The plants can grow up to 15 centimeters in length, have a main axis with alternate plumed branches and are usually attached to rocks in shallow rocky-bottomed water with high luminosity (Figure 1.2.).

Although it is not usually part of the human diet, this seaweed can be harvested on a smaller scale as a food supplement and for pharmaceutical applications, since it contains various antimicrobial substances (Munda, 2006) and is also known to be an ingredient in personal care products.

Figure 1.2. – *Halopteris scoparia* attached to rocks, Azores islands, Atlantic Ocean.
SeaExpert, 2017.

1.3.3. *Petalonia binghamiae*

Petalonia binghamiae (J. Agardh) is a brown macroalgae (Phaeophyceae) commonly used as food in China, Japan, Taiwan and Korea. It is very widely distributed thus it can be found all the way from the Atlantic to the Pacific (Mori et al., 2004, Yang et al., 2010).

The physiognomy of this seaweed (Figure 1.3.) can be described as an aggregate of leaves that are 20-30 mm in width and around 250 mm in length, though it can grow up to 13 cm long (Pereira, 2016). There are studies that suggest *P. binghamiae* extracts possess anti-diabetic (Kang et al., 2008), anti-allergic (Kimiya et al., 2008), antioxidant (Kuda et al., 2006) and anti-inflammatory activities (Yang et al., 2010).

Figure 1.3. – Freshly caught exemplar of *Petalonia binghamiae*, Azores islands, Atlantic Ocean.
SeaExpert, 2017.

2. Objective

Marine resources, in particular seaweeds, have drawn significant attention from the scientific community due to their vast properties and possible application, both as functional food and nutraceutical products. Many studies have been conducted in the past years, but macroalgae are organisms so diverse that there are still many species whose composition and properties are virtually unknown. Even among algae of the same species, simple factors such as their geographical location can affect their composition and properties.

In Portugal, the interest for these organisms has also been growing and several institutes have joined efforts to obtain a better characterization of the marine macroalgae that can be found here. Some Azorean species are of particular interest, since they are already being regularly collected and commercialized: *Halopteris scoparia*, *Petalonia binghamiae* and *Osmundea pinnatifida.* The last two species, after sun-drying, are already used for culinary purposes in some Azorean islands.

The objective of this work was to conduct a bioprospection of the sun-dried biomass of these three undervalued and not well-known Azorean macroalgae. This involved their chemical characterization by determination of their proximate composition, lipid and fatty acid profile, total phenolic content and β-glucans (laminarin) levels. Some of the most relevant bioactivities were also determined, such as antioxidant activity (measured by DPPH, FRAP, and ABTS methods) and anti-inflammatory activity, thus establishing a knowledge basis for the development of future applications.

3. Materials and methods

3.1. Sample collection and preparation

The studied seaweed species were *Petalonia binghamiae*, *Halopteris scoparia*, and *Osmundea pinnatifida*, being the first two brown seaweeds, from Ectocarpales order (Scytosiphonaceae family) and Sphacelariales order (Stypocaulaceae family), respectively, and the last one a red seaweed belonging to the Ceramiales order and Rhodomelaceae family (Pereira 2016). They were all from the Azores islands (located in the mid-North Atlantic) —more specifically, Faial island. *P. binghamiae* and *O. pinnatifida* were collected from the wild in March 2017, *H. scoparia* in October 2017. These seaweeds were supplied to the Portuguese Institute of the Sea and Atmosphere (IPMA) by the Portuguese company seaExpert (Faial, Azores, Portugal) after a specialist in phycology ensured a correct taxonomic identification. Frond seaweed biomass was sun-dried, packaged in plastic bags, and sent to IPMA laboratory at Lisbon (Figure 3.1.). Afterwards, adequate amounts of each macroalgae were freeze-dried and stored at -80 °C until further analysis.

Figure 3.1. Sun-dried seaweed biomass of the three studied species: *Osmundea pinnatifida* **(O)**, *Halopteris scoparia* **(H)** and *Petalonia binghamiae* **(P)**.
Ana Campos, 2017.

3.2. Proximate composition

3.2.1. Moisture and ash

Moisture and ash contents were determined according to AOAC methods (AOAC 2000). These parameters indicate, respectively, the amount of water and inorganic material present in the samples. Briefly, 2g of each macroalgae were weighed in duplicate to crucibles that were previously heated in the oven (100°C for 30 minutes) and properly weighed after cooling. Samples were then left in the oven overnight and weighed in the next day after cooling. The moisture content of each sample was calculated according to the formula:

$$\%\, Moisture\ \left(\%\frac{m}{m}\right) = \frac{m1 - m2}{m1 - m3} \times 100 \qquad \textbf{(3.1.)}$$

Where:
m1 = Crucible mass with moist sample (g)
m2 = Crucible mass with dry sample (g)
m3 = Crucible mass (g)

The crucibles with the dried samples were then placed in the lab furnace at 550°C for 24 hours (Figure 3.2.). After this period the crucibles were removed and allowed to cool for 40 minutes, weighed, and again placed in the furnace for another 30 minutes at the same temperature. At last the crucibles were again removed, allowed to cool and weighed. If the weight has increased since the first weighing it is not necessary to repeat this process again. The ash content of each sample was calculated according to the formula:

$$\%Ash\ \left(\%\frac{m}{m}\right) = \frac{m1 - m2}{m3 - m2} \times 100 \qquad \textbf{(3.2.)}$$

Where:
m1 = Crucible mass with ash (g)
m2 = Crucible mass (g)
m3 = Crucible mass with dry sample (g)

Figure 3.2. - Crucibles with ash at the end of the procedure.
Ana Campos, November 2017.

3.2.2. Protein content

The protein level was quantified according to the Dumas method (Saint-Denis and Goupy 2004) and a conversion factor of nitrogen into protein of 5.5 was used. This method consists of combusting a sample of known mass in the presence of oxygen inside a high temperature chamber (900°C), which allows to calculate the amount of nitrogen in it based on its thermal conductivity.

To do this, 120g of each seaweed were weighed in duplicate and placed in a LECO FP-528 analyzer which performed the entire combustion process and calculated the nitrogen percentage and the corresponding protein percentage for each sample. Ethylenediamine tetraacetic acid (EDTA) was used to calibrate the standards.

3.2.3. Total lipid content

The total lipid content in macroalgae is usually attained by using one of two distinct extraction methods: Bligh and Dyer (1959) or Folch (Folch et al., 1957). Since there is no consensus in the scientific community about which method is most effective for marine algae due to their biological variability, both were performed and the results were subsequently analyzed and compared with existing literature to understand which one was more efficient (see Annex II).

Bligh and Dyer method

Briefly, 1g of each seaweed was weighed in triplicate into 20ml tubes and homogenized with 5ml of methanol: chloroform solution (2:1) solution using a Polytron PT 6100 model for 1 minute. Subsequently, 1ml of saturated sodium chloride solution, 2ml of chloroform and 2ml of purified water were sequentially added and homogenized for 30 seconds. The homogenized samples were centrifuged (10 minutes, 4°C, 2000×g), the organic phase was collected into an 18ml tube with anhydrous sodium sulfate and centrifuged again to remove any residual water that might still be present in the sample. The organic phase was then filtered through a column filled with cotton and anhydrous sodium sulfate to a previously weighed pear-shaped flask, being this column washed twice with 2ml of chloroform in order to guarantee that no lipids were retained. The chloroform was evaporated in an RE 121 model rotary evaporator (Büchi, Flawil, Switzerland) with the water bath temperature at 45°C. Finally, the flask with the lipids was reweighted and the total lipid percentage in each sample was calculated using the following equation:

$$Total\ lipids\ (\%) = \frac{Mf - Mi}{Ms}\ x\ 100 \qquad \textbf{(3.3.)}$$

Where:
Mf = Pear-shaped flask mass with lipids (final) (g)
Mi = Pear-shaped flask mass (initial) (g)
Ms = Sample mass (g)

Folch method (adapted)

In this method 200mg of each macroalgae were weighed in triplicate and mixed with 3ml of chloroform: methanol (2:1) solution. The tubes were placed in a shaking water bath (25°C, 400 rpm) for 10 min and then 3ml of hydrochloric acid and 300µl of magnesium chloride were added. The samples were centrifuged (5 minutes, 4°C, 3000×g) and the organic phase was collected and filtered through a column filled with cotton and anhydrous sodium sulfate to another previously weighed tube, being this process repeated to ensure the extraction of as many lipids as possible. The filtration step is not included in the original method, but it was necessary to avoid the presence of seaweed residue and water in the collected organic phase, since these can severely interfere with the final sample mass and lead to a wrong calculation of the lipid content. Then, 3ml of chloroform: methanol (2:1) solution were added to all initial tubes, which were then placed again in the shaking water bath for 5 minutes and centrifuged (5 minutes, 4°C, 2000×g).

The new organic phase was collected and added to the corresponding phase previously collected. To evaporate the solvents the tubes were placed in the oven for 24 hours and then weighed. The total lipid content was calculated using the equation 3.3., but:

Mf = Tube mass with lipids (final)
Mi = Tube mass (initial)
Ms = Sample mass

3.2.4. Carbohydrate content

Carbohydrate content was determined by difference between 100 % and the sum of the moisture, protein, crude fat, and ash contents.

3.3. Lipid classes

The main lipid classes were separated by analytical thin-layer chromatography (TLC) in plates coated with 0.25mm silica gel G based on the method described by Bandarra et al. (1997).
Lipids extracted from the *Petalonia binghamiae*, *Halopteris scoparia*, and *Osmundea pinnatifida* using the adapted Folch method (3.2.3) were dissolved in chloroform (10mg/ml concentration). Glyceryltrioleate (TAG), 1,2 and 1,3-diacylglycerol (DAG-1,2 and DAG-1,3), free fatty acid (FFA), cholesterol (CH), L-α-phosphatidylcholine (PL) and psychosine (PSY) standards (Sigma Chemical Co., St. Louis, Mo) were also prepared in chloroform at the same concentration.

In order to correctly separate the main lipid classes (TAG, DAG, FFA, CH and PL), two distinct elution mixtures were used: hexane, diethyl ether and acetic acid (65:35:1 by volume) and hexane, diethyl ether and acetic acid (50:50:1 by volume). The first mixture separated almost all classes but is was verified the co-elution of DAG and sterols, so the second mixture was used to separate these classes and allow their correct quantification in the samples (Figure 3.3.A e B). To attempt to separate the polar lipid (PL) fraction (the phospholipids from the glycolipids) a third elution mixture was tested using phospholipid and glycolipid standards: chloroform: methanol: acetic acid and ultra-pure water (85:20:10:4 by volume) (Wang and Benning 2011). It was verified a significant amount of drag in the standards (PL and PSY) with the initial proportions, so the mixture was optimized to a final ratio of 70:20:10:4 by volume (Figure 3.3.C.). After each plate was properly identified, the samples and standards (10µL in the corresponding well) were applied in duplicate to the plates and each plate was immersed in the respective elution mixture inside a developing chamber. After the elution front reached the upper limit, the plates were taken from the chamber, allowed to dry for 10 minutes, and then sprayed with 10% phosphomolybdic acid in ethanol (w/v). Finally, the plates were placed in an oven at 110ºC for one hour to reveal. Identification of lipid classes was done by visual comparison with the standards. Quantification was performed using a scanner and version 4.5.2 of Quantity One 1-D Analysis software from Bio-Rad (Hercules, CA, USA). This equipment allowed to calculate the relative percentages of the different identified lipid classes based on the area of the spots pondered by the optical density on the TLC plate (Figure 3.3.D).

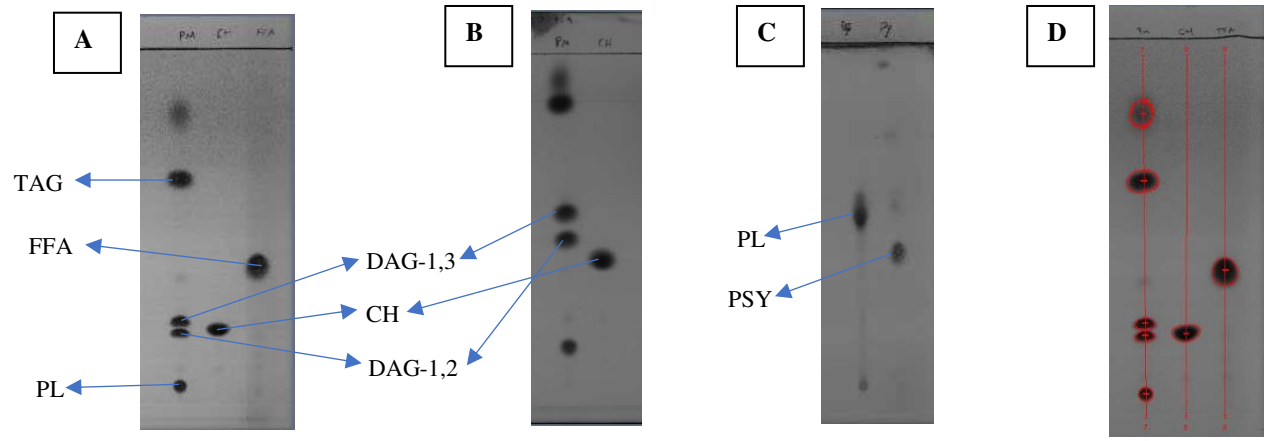

Figure 3.3. Separation of lipid classes by thin layer chromatography (TLC). With an hexane, diethyl ether and acetic acid (65:35:1 by volume) mixture **(A)**, there is co-elution of the DAG and CH standards. The proportion 50:50:1 by volume was used to achieve their separation **(B)**. A mixture of chloroform, methanol, acetic acid and ultra-pure water mixture (70:20:10:4 by volume) was used to separate the polar lipid fraction, that is to separate the phospholipids (PL) from the glycolipids (PSY) **(C)**. The different classes were then quantified based on the area of the corresponding spots **(D).**
Ana Campos, December 2017/January 2018.

3.4. Fatty acid profile

Fatty acid methyl esters (FAME's) were prepared from the freeze-dried seaweed by acid-catalysed transesterification using the methodology described by Bandarra et al. (1997).

Succinctly, 300mg of each lyophilized macroalgae was weighed to 15ml tubes, mixed with 5ml of acetyl chloride: methanol (1:19) mixture, vortexed for 30 seconds, and placed in a water bath (80°C) for one hour. After cooling for 30 minutes, 1ml of ultra-pure water and 2ml of n-heptane were added to each tube.The tubes were centrifuged (3 minutes, 4°C, 3000×g) and the organic phase was collected to a 2ml vial, through a filter of cotton and anhydrous sodium sulfate. Samples were then applied to a DB-WAX (Agilent Technologies, Santa Clara, USA) capillary column (film thickness, 0.25µm, 30m × 0.25mm i.d.), integrated in a Varian Star 3800 CP gas chromatograph (Walnut Creek,CA, USA), equipped with an auto sampler with a split injector (100:1) and a flame ionization detector, both at 250 °C. The separation of the FAMEs was carried out with helium as the carrier gas and using a temperature program for the column starting at 180ºC and increasing to 200°C at 4°C/minute, holding for 10 minutes at 200°C, heating to 210°C at the same rate, and holding at this temperature for 14.5 minutes. FAME's were identified by comparing their retention time with those of Sigma–Aldrich standards (PUFA-3, Menhaden oil, and PUFA-1, Marine source from Supelco Analytical). The limit of detection was 1mg/100g. Results were calculated in mg/100g of edible part using an internal standard (17:0). Analyses were always done in triplicate.

3.5. Beta-glucans (laminarin) content

Laminarin in the seaweed samples was quantified by measuring the glucose concentration released after enzymatic hydrolysis of laminarin. This was achieved through the utilization of the β-glucan assay kit supplied by Megazyme (Bray, Ireland).

Briefly, 120mg of each sample was weighed in triplicate and mixed with 200µl of ethanol 50% and 4ml of phosphate buffered saline (PBS) (20mM, pH 6.5). Immediately after adding the PBS, the tubes were placed in a boiling water bath for 3 minutes and incubated at 50°C for 5 minutes. Then, 200µl of lichenase enzyme were added and the tubes were left to incubate during one hour in a bath (50°C) with regular shaking. The samples were mixed with 5ml of sodium acetate buffer (20mM, pH 4.0), centrifuged (5 minutes, 4°C, 1000×g) and three 100µl aliquots were done to each tube. To the first and second aliquot, 100µl of β-glucosidase enzyme were added. The third one was mixed with 100µl of buffer (50mM, pH 4.0) and will act as a blank, because β-glucosidase is not present to catalyse the hydrolysis of the glycosidic bonds. The aliquots were all incubated at 50°C for 10 minutes and after incubation, 3 ml of GOPOD (glucose oxidase/peroxidase) reagent was added. This mixture was then incubated at 50°C for 20 min. The absorbance of the samples was measured at 510 nm in a Helios Alpha model (Unicam, Leeds, UK). β-glucan from oats supplied also in the kit was used as a standard (Figure 3.4.). Results were expressed in %, w/dw according to the formula supplied by the assay kit:

$$\beta glucans\ \left(\%\frac{w}{w}\right) = \Delta Abs \times \frac{F}{W} \times \mathrm{FV} \qquad \textbf{(3.4.)}$$

Where:

ΔAbs = Absorbance after β-galactosidase treatment (reaction) minus reaction blank absorbance.
F = Factor for the conversion of absorbance values to µg of glucose =
= 100 (µg of D-glucose) / absorbance of 100µg of D-glucose
W = sample mass (mg)
FV = Final volume (i.e. 9.4ml)

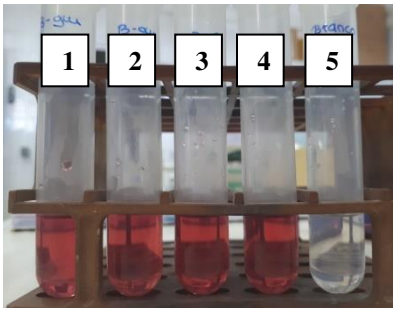

Figure 3.4. – Coloring of the positive control tubes (**1** and **2**), the D-glucose standard (**3** and **4**) and the blank (**5**) after β-galactosidade treatment. The pink colour in the mixture indicated the presence of D-glucose, and therefore the presence of β-glucans.
Ana Campos, May 2018.

3.6. Total polyphenol content

3.6.1 Extract preparation

Phenolic compounds were extracted from the seaweed biomass by selected solvents - water or ethanol 96 %, w/w. These solvents were chosen among different safe and environmentally acceptable solvents (also including 2-butanol, ethyl acetate and isoamyl acetate) as being the most effective.

In order to prepare the extracts, 1.25g of dried seaweed biomass was weighed, homogenized with 25ml of water or ethanol 96 %, w/w, using a model Polytron PT 6100 homogenizer (Kinematica, Luzern, Switzerland) at a velocity of 30,000 rpm during 1 minute, and agitated for 18 hours on an orbital shaker. After centrifugation (5000×g at room temperature during 20 minutes), the supernatant was collected through a filter to a final volume of 25ml.

3.6.2. Singleton and Rossi assay

Total polyphenol content was determined by an adapted version of the Singleton and Rossi method using the Folin-Ciocalteu reagent (Singleton and Rossi, 1965; Ripol et al. 2018). This reagent is yellow in its non-reduced form, but when reduced by the presence of phenolic compounds turns blue and therefore allows their quantification.

A volume of 100µl of each seaweed extract was added to a vial. To each vial, 600µl of ultra-pure water plus 150µl of twice-diluted Folin-Ciocalteau reagent were added and allowed to stand for 5 minutes at room temperature. Then, 750µl of a 2% w/v sodium carbonate solution were added. The original assay indicates that the samples should be allowed to react in the dark for only 15 minutes, but since no change in the coloration occurred at the end of this time, other reaction times were tested: 45 minutes, 1h and 30 minutes and 3 hours. Up to 1h30min the reaction seems to still be occurring, stabilizing after this period. So, after being left to react for 1 hour and 30 minutes in the dark at room temperature, absorbance of the samples at 750 nm was measured in a Helios Alpha model (Unicam, Leeds, UK) UV-Vis spectrophotometer Gallic acid (GA) was used as standard and phenolic content was expressed as gallic acid equivalents (mg GAE/100g) through the calibration curve of gallic acid (Sigma, Steinheim, Germany) (see Annex III).

3.7. Antioxidant activity

3.7.1. DPPH method

The antioxidant activity was measured through the determination of the radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Miliauskas et al., 2004). DPPH is considered a stable free radical because the spare electron is delocalized, which means the molecules do not dimerize like most free radicals. This characteristic also gives DPPH a deep violet colour, with an absorption around 520nm. When a substance capable of donating an hydrogen atom is mixed with DPPH, its reduced form shows a less violet colour, gradually becoming yellowish (Figure 3.5.). This method usually offers the first approach for evaluating the antioxidant potential of a compound or an extract (Kedare and Singh, 2011).

To perform this analysis, a volume of 1 ml of the extract (as prepared in 3.6.1) was added in triplicate for each sample and 2ml of DPPH (Sigma, Steinheim, Germany) 0.15 mM methanolic solution was added and thoroughly mixed. After 30 minutes of incubation at room temperature in the dark, absorbance was measured at 517nm in a Helios Alpha model (Unicam, Leeds, UK) UV/visible light spectrophotometer. Either water or ethanol 96%, w/w, was used as the blank. Radical scavenging activity was calculated by the following formula:

$$\% \, Inhibition = \frac{A0 - Asample}{A0} \times 100 \qquad \textbf{(3.5.)}$$

Where:
A0 – Absorbance of the blank.
Asample – Absorbance of the sample.

Results were expressed in mg of ascorbic acid equivalents (AA Eq) per l.

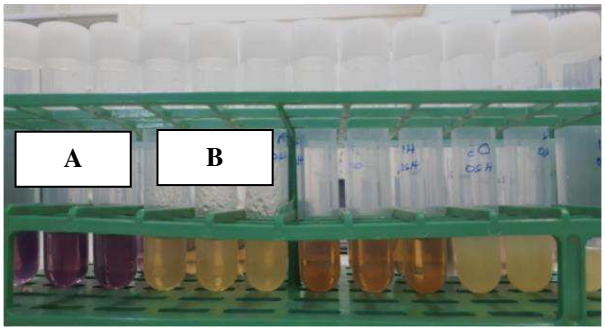

Figure 3.5. – Colour variation during the DPPH reaction. DPPH has a characteristic violet colour **(A)** but when mixed with an antioxidant substance (capable of donating an hydrogen atom) gradually becomes yellow **(B).**
Ana Campos, March 2018.

3.7.2. FRAP method

The applied Ferric Ion Reducing Antioxidant Power (FRAP) method was a modified technique based on Benzie and Strain (1996). As described by the authors, this method is based on redox reactions where the oxidizing species reacts with the antioxidant instead of the substrate, meaning that the antioxidant (reductant) reduces the oxidant. Therefore, if an easily reduced oxidant is present in excess, the antioxidant ability of a certain reductant can easily be assessed, especially if there is colour change associated with the reaction. In this specific case, when a ferric-tripyridyltriazine (Fe^{III}-TPTZ) complex is reduced to the Fe^{II} form it exhibits an intense blue colour (Figure 3.6.). This means that in FRAP method Fe^{III} is used in excess and what limits the formation of Fe^{II} is the reducing ability of the sample in study. To perform this reaction, the stock solutions used were 300mM acetate buffer (pH 3.6), 10mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40mM HCl, and 20mM $FeCl_3 \bullet 6H_2O$ solution. The fresh working solution was prepared by mixing 25ml acetate buffer, 2.5ml TPTZ, and 2.5ml $FeCl_3 \bullet 6H_2O$.

A volume of 100µl of sample (prepared in 5%, w/v, aqueous and ethanolic extracts attained as described in section 3.6.1.) was allowed to react with 3ml of the FRAP solution for 30 min in the dark at 37 °C. Readings of the colored product (ferrous-tripyridyltriazine complex) were taken at 595 nm. The standard curve was linear between 250 and 2000µM $FeSO_4$ (see Annex IV). Results were expressed in mM Fe^{II} and compared with an ascorbic acid control.

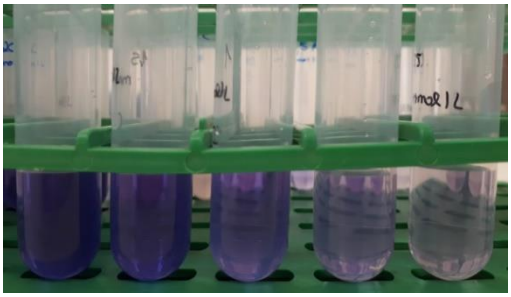

Figure 3.6. – Colour variation in the FRAP reaction. The intense blue colour means that the Fe^{III}-TPTZ complex was reduced, indicating the presence of antioxidant activity.
Ana Campos, March 2018

3.7.3. ABTS method

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) radical scavenging activity was determined using the method described by Re et al. (1999). In this assay, by addition of sodium persulfate, ABTS is converted to its radical cation that is blue in color and allows the reaction to be monitored by spectrophotometry (Figure 3.7.). This required the preparation of the method's specific reagent (7 mM $ABTS^{+\bullet}$ solution) as follows: 10mg of ABTS was dissolved in 2.6ml of a 2.45-mM potassium persulfate solution. The solution remained for 16 hours in the dark at room temperature before use and the $ABTS^{+\bullet}$ solution was diluted with 5mM sodium phosphate buffer (pH 7.4) to give an absorbance value of 0.70 ± 0.02 at 734nm. Then, 20µl of sample solutions (prepared in 5%, w/v, aqueous and ethanolic extracts attained as described in section 3.6.1.) were added to 2ml of the diluted $ABTS^{+\bullet}$ solution and the mixture was homogenized and incubated in the dark at 30°C for 6 minutes. The absorbance of the samples was measured at 734nm in a Helios Alpha model (Unicam, Leeds, UK). The ABTS radical scavenging activity of the samples was expressed as percentage of inhibition:

$$\%\ Inhibition = \frac{A0 - Asample}{A0} \times 100 \qquad \textbf{(3.6.)}$$

Where:
A0 – Absorbance of the blank.
Asample – Absorbance of the sample.

Results were expressed in µmol of trolox equivalents (Trolox Eq) per g dw of seaweed.

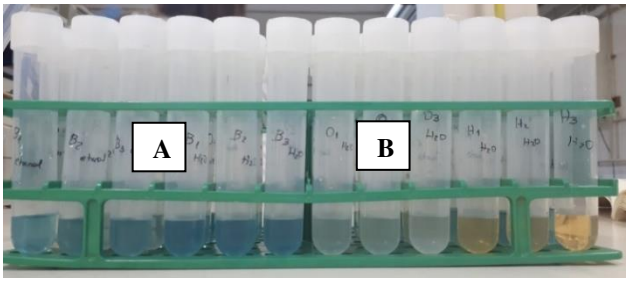

Figure 3.7. – Colour variation in the ABTS reaction. The $ABTS^{+}$ solution exhibits a blue colour **(A)** and the loss of this colour indicates the presence of antioxidant agents **(B)**.
Ana Campos, April 2018

3.8. Anti-inflammatory activity

3.8.1. Extract preparation for in vitro anti-inflammatory activity

The anti-inflammatory activity of *Petalonia binghamiae*, *Halopteris scoparia*, and *Osmundea pinnatifida* was determined in aqueous and alcoholic extracts.

Approximately 200mg of sun-dried and freeze-dried seaweed was weighed and homogenized with 2ml of ultra-pure water or ethanol 96% w/w, using a model Polytron PT6100 homogenizer (Kinematica, Luzern, Switzerland) at a velocity of 30 000 rpm during 1 min. The mixtures were subjected to heat treatment (80ºC for 1 hour) and then centrifuged (10 minutes, 4ºC, 3000×g). The supernatant was collected and the solvent was evaporated using a vacuum rotary evaporator with the water bath temperature at 65°C. The residue was directly dissolved in 100% dimethyl sulfoxide (DMSO) to prepare a stock solution with a concentration of 10mg/ml.

3.8.2. Cyclooxygenase (COX-2) inhibition method

Cyclooxygenase (COX) is a bifunctional enzyme that converts arachidonic acid to a hydroperoxyl endoperoxide (PGG_2) and reduces the endoperoxide to the corresponding alcohol (PGH_2), which is the precursor of prostaglandins (PGs), thromboxanes and prostacyclins (Hamberg and Samuelsson, 1973). The COX inhibitor screening assay directly measures the amount of Prostaglandin F2α generated from arachidonic acid (AA, 20:4 ω6) in the cyclooxygenase reaction, being a practical and relatively quick screening method for assessing the anti-inflammatory activity.

The seaweed extracts were tested at 1 mg/ml using a commercial cyclooxygenase (COX) inhibitory screening assay kit, Cayman test kit-560131 (Cayman Chemical Company, Ann Arbor, MI, USA). A volume of 10µl each extract or DMSO was used. The reaction was initiated by addition of 10µl 10mM AA and each reaction tube was incubated at 37°C for 2 minutes. It was terminated by addition of 50µl 1 N HCl and saturated stannous chloride.

Assays were performed using 100 units of human recombinant COX-2. An aliquot was removed and the prostanoid produced was quantified spectrophotometrically (412nm) via enzyme immunoassay (ELISA) after an 18-hour incubation, washing, addition of Ellman's reagent, and further 90-minute incubation (Figure 3.8.). Results were expressed as a percentage of inhibition of COX-2.

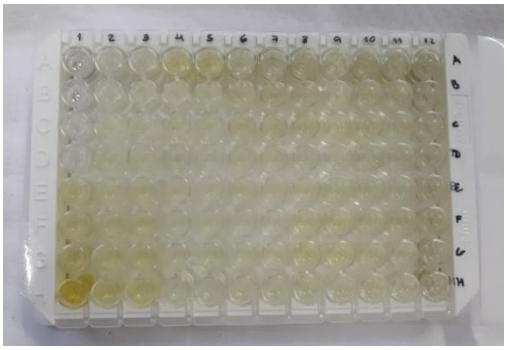

Figure 3.8. – Anti-inflammatory activity test plate ready for prostanoid reading.
Ana Campos, March 2018.

3.9. Statistical analysis

To test the normality and the homogeneity of variance of data, the Kolmogorov-Smirnov's test and Levene's F-test, respectively, were used. Data which corroborated these assumptions were analysed by an one-way ANOVA distribution using the Tukey HSD to determine the difference in the constituents contents between seaweed species or by a factorial ANOVA using the Tukey HSD to determine the difference in biological activities between species and between aqueous and ethanolic extracts. When normality and/or homogeneity of variance were not verified (18:2 ω6 content), data were tested non-parametrically with Kruskal-Wallis test (analysis of variance) followed by nonparametric multiple comparisons test (Zar, 1999). For all statistical tests the significance level (α) was 0.05. All data analysis was performed using STATISTICA 6 (Stat-sof, Inc. USA, 2003).

4. Results

4.1. Proximate composition

The final proximate composition of all studied seaweed species is displayed in Table 4.1. Some differences between species can easily be observed.

Regarding moisture, all values were relatively low, being *H. scoparia* the one that exhibited the lower amount (3.2% dw). The ash content was particularly high in this same macroalga (51.9% dw). The protein content was the parameter where there was less variation between species. Lipid content was low for all samples, but *P. binghamiae* showed values significantly higher than the other two species (4.5% dw). This seaweed was also the one with higher carbohydrate content (48.3% dw). A visual representation of the relative percentages of each component for each seaweed is depicted in Figure 4.1.

Table 4.1. - Proximate crude composition (g/100 g dry weight for ash, protein, fat, and carbohydrate) in the three studied seaweed species. Values are presented as average ± standard deviation. Different lowercase letters within a row correspond to statistical differences (p<0.05) between seaweeds.

	Petalonia binghamiae	***Halopteris scoparia***	***Osmundea pinnatifida***
Moisture	6.7 ± 0.0^{b}	3.2 ± 0.1^{a}	6.7 ± 0.2^{b}
Ash (g/100 g dw)	27.9 ± 0.0^{a}	51.9 ± 0.1^{c}	39.4 ± 0.4^{b}
Protein (g/100 g dw)	12.6 ± 0.3^{b}	10.3 ± 0.1^{a}	14.4 ± 0.0^{c}
Lipid (g/100 g dw)[1]	4.5 ± 0.3^{c}	1.9 ± 0.1^{b}	1.6 ± 0.1^{a}
Carbohydrate (g/100 g dw)	48.3^{c}	32.5^{a}	37.9^{b}

[1] - Extracted by the Folch method.

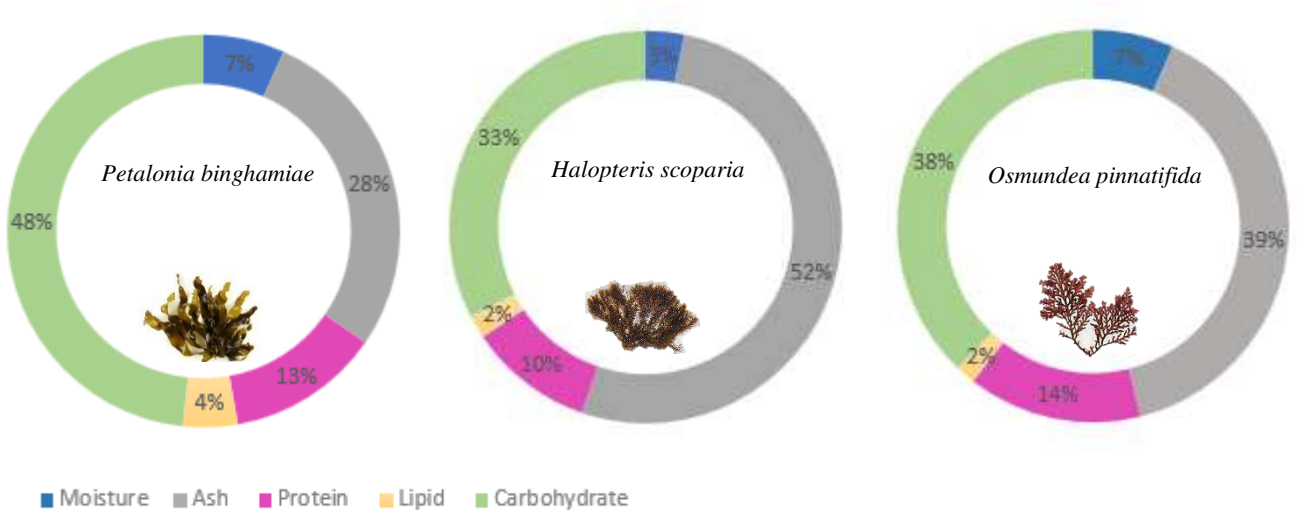

Figure 4.1. – Proximal composition of each studied seaweed species, represented in percentage.

4.2. Lipid classes

The lipid class distribution in the TLC plates for each seaweed using the hexane, diethyl ether and acetic acid (65:35:1 by volume) elution mixture is exemplified in Figure 4.2. This mixture allowed the separation of non-polar lipids from polar lipids. The non-polar fraction includes triacylglycerols (glyceryltrioleate -TAG), free fatty acids (FFA), diacylglycerols (DAG-1,2 and DAG-1,3) and sterols (CH). The polar fraction is composed by phospholipids and glycolipids, which with this mixture co-elute very close to the application point and are both quantified together using the PL standard. The quantification of each separated class is shown in Table 4.2. The non-polar fraction was significantly higher than the polar fraction for all species. TAG was the most abundant class of lipids in the three studied macroalgae, being the highest value attained for *Petalonia binghamiae* (46.9%). FFA content was similar for all seaweeds, varying from 27.9% in *Osmundea pinnatifida* to 33.1% in the other two species. DAG + CH levels were higher in *O. pinnatifida* (19.2%). Regarding the brown macroalgae, *H. scoparia* exhibited a slightly higher amount of DAG + CH than *P. binghamiae* (12.8% and 12.1%, respectively). The polar lipid fraction was very variable between seaweeds, going from 8.7% *in P. binghamiae* up to 21.1% in *O. pinnatifida*.

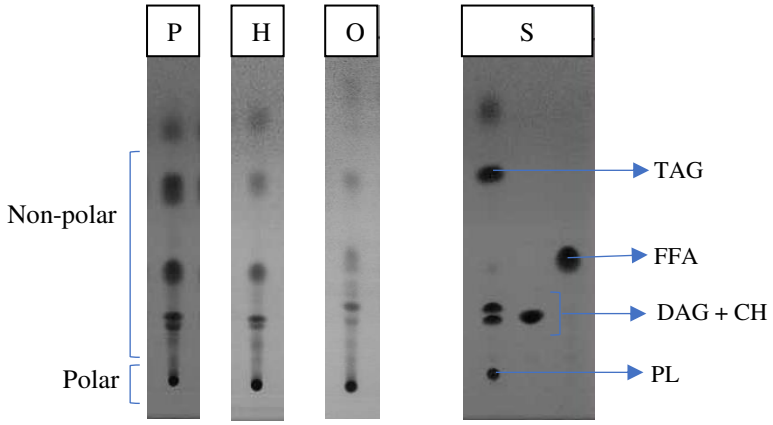

Figure 4.2. - Lipid class distribution in the TLC plates for *Petalonia binghamiae* **(P)**, *Halopteris scoparia* **(H)**, *Osmundea pinnatifida* **(O)** and the standards **(S)** using the hexane, diethyl ether and acetic acid (65:35:1 by volume). The polar lipid fraction (PL) stays very close to the application point. The non-polar fraction includes the TAG, FFA, DAG and CH classes. The standards are: PL, DAG-1,2, CH, DAG-1,3, FFA and TAG.

Table 4.2. – Lipid classes attained by TLC, using an elution mixture of hexane, diethyl ether and acetic acid (65:35:1 by volume). Relative percentages of glyceryltrioleate (TAG), free fatty acid (FFA), diacylglycerol (DAG), sterol (CH) and L-α-phosphatidylcholine (PL) were obtained for all seaweed species. 1,3-diacylglycerol (DAG-1,3), 1,2-diacylglycerol (DAG-1,2) and sterol (CH) co-eluted and are quantified together. Values are presented as average ± standard deviation. Different lowercase letters within a row correspond to statistical differences (p<0.05) between seaweeds.

	Standards	*Petalonia binghamiae*	*Halopteris Scoparia*	*Osmundea pinnatifida*
Non-polar	**TAG**	46.9 ± 1.8^{c}	36.7 ± 2.7^{b}	31.7 ± 2.5^{a}
	FFA	33.1 ± 2.4^{b}	33.1 ± 2.8^{b}	27.9 ± 3.0^{a}
	DAG + CH	12.1 ± 0.6^{a}	12.8 ± 0.6^{b}	19.2 ± 1.4^{c}
Polar	**PL**	8.7 ± 0.6^{a}	17.3 ± 1.1^{b}	21.1 ± 2.9^{c}

To separate DAG from CH and thus quantify the diacylglycerol and sterol classes, an hexane, diethyl ether and acetic acid (50:50:1 by volume) elution mixture was used. The lipid class distribution in the TLC plates for each seaweed using this mixture is shown in Figure 4.3., and the corresponding quantification, crossed with the data from the previously used mixture (DAG + CH), is presented in Table 4.3. Standards of DAG-1,2, DAG-1,3 and CH were used, but although there is a spot relatively close to the zone where DAG-1,3 should elute, there is a considerable deviation when compared to the standard for all macroalgae, so this class cannot be visually identified as DAG-1,3 with certainty and therefore has been quantified as unidentified (Ni). DAG-1,2 content was very low in all samples, specially in *O. pinnatifida* (1.7% of the total lipid classes). Sterol content was higher in *O. pinnatifida* (5.9%) and the lowest value was obtained for *P. binghamiae* (2.6%).

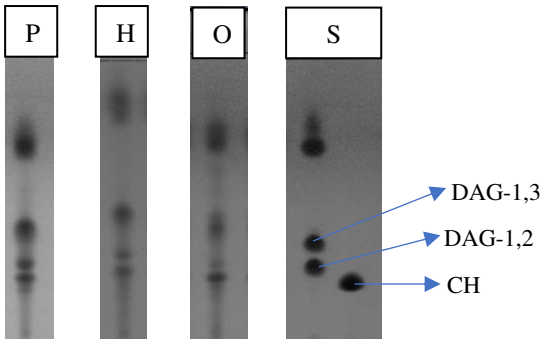

Figure 4.3. - Lipid class distribution in the TLC plates for *Petalonia binghamiae* **(P)**, *Halopteris scoparia* **(H)**, *Osmundea pinnatifida* **(O)** and the standards **(S)** using the hexane, diethyl ether and acetic acid (50:50:1 by volume). This mixture successfully separates DAG-1,2 from sterols (CH). It was not possible to identify DAG-1,3 with certainty in the samples.

Table 4.3. - Lipid classes attained by TLC, using an elution mixture of hexane, diethyl ether and acetic acid (50:50:1 by volume). Relative percentages of sterols (CH) and 1,2 diacylglycerol (DAG-1,2) were obtained for all seaweed species.
Ni = Unidentified. Values are presented as average ± standard deviation. Different lowercase letters within a row correspond to statistical differences (p<0.05) between seaweeds.

	Petalonia binghamiae	***Halopteris scoparia***	***Osmundea pinnatifida***
CH	2.6 ± 0.4^{a}	3.6 ± 0.2^{b}	5.9 ± 0.7^{c}
DAG - 1,2	2.1 ± 0.2^{b}	2.0 ± 0.3^{b}	1.7 ± 0.5^{a}
Ni	7.3 ± 0.4^{a}	7.2 ± 0.3^{a}	11.6 ± 1.2^{b}

Finally, in order to attempt to separate the polar lipid fraction (the phospholipids from the glycolipids) a third elution mixture was used: chloroform: methanol: acetic acid and ultra-pure water (70:20:10:4 by volume). The separation was successfully achieved between the standards (L-α-phosphatidylcholine and psychosine), but in the samples there was no visible glycolipid (PSY) fraction and even the phospholipid fraction (PL) was very subtle, so it was not possible to quantify these classes.

4.3. Fatty acid profile

The FA profile (in % of total FAs and in mg/100 g dry weight) of the studied seaweed species *Petalonia binghamiae*, *Halopteris scoparia*, and *Osmundea pinnatifida* is shown in Table 4.4.

Table 4.4. - Fatty acid profile (in % of total fatty acids and in mg/100 g dry weight) of the studied seaweed species *Petalonia binghamiae*, *Halopteris scoparia*, and *Osmundea pinnatifida*. Values are presented as average ± standard deviation. nd – not detected. Different lowercase letters within a row correspond to statistical differences (p<0.05) between the relative (%) FA profiles of seaweeds. Different uppercase letters within a row correspond to statistical differences (p<0.05) between the absolute (mg/100 g dw) FA profiles of seaweeds.

Fatty acid	***Petalonia binghamiae***		***Halopteris scoparia***		***Osmundea pinnatifida***	
	(% total fatty acids)	(mg/100 g dw)	(% total fatty acids)	(mg/100 g dw)	(% total fatty acids)	(mg/100 g dw)
14:0	6.6 ± 0.1^{a}	114 ± 2^{B}	7.5 ± 0.1^{b}	52 ± 1^{A}	8.8 ± 0.0^{c}	59 ± 3^{A}
16:0	24.8 ± 0.1^{a}	427 ± 10^{C}	32.6 ± 0.3^{b}	225 ± 6^{A}	47.2 ± 0.1^{c}	315 ± 12^{B}
18:0	1.0 ± 0.0^{a}	17 ± 1^{C}	1.8 ± 0.0^{b}	12 ± 0^{B}	1.0 ± 0.1^{a}	7 ± 0^{A}
Σ SFA	38.5 ± 0.2^{a}	665 ± 17^{C}	41.9 ± 0.4^{b}	289 ± 8^{A}	57.0 ± 0.1^{c}	381 ± 15^{B}
16:1 ω9	1.4 ± 0.0^{a}	24 ± 1^{B}	1.9 ± 0.1^{b}	13 ± 0^{A}	2.2 ± 0.1^{c}	15 ± 0^{A}
16:1 ω7	1.3 ± 0.1^{b}	23 ± 1^{C}	2.7 ± 0.0^{c}	18 ± 1^{B}	nd^{a}	nd^{A}
18:1 ω9	17.0 ± 0.1^{c}	293 ± 6^{C}	7.9 ± 0.1^{a}	54 ± 2^{A}	12.1 ± 0.0^{b}	81 ± 3^{B}
18:1 ω7	0.9 ± 0.0^{a}	16 ± 1^{B}	1.5 ± 0.1^{b}	10 ± 1^{A}	4.3 ± 0.0^{c}	29 ± 1^{C}
Σ MUFA	20.6 ± 0.1^{c}	356 ± 9^{C}	13.9 ± 1.0^{a}	96 ± 2^{A}	18.6 ± 0.1^{b}	124 ± 4^{B}
18:2 ω6	7.1 ± 0.0^{b}	123 ± 3^{C}	13.4 ± 0.1^{c}	92 ± 2^{B}	1.3 ± 0.0^{a}	9 ± 1^{A}
20:4 ω6	7.9 ± 0.1^{b}	136 ± 4^{C}	9.2 ± 0.2^{c}	63 ± 2^{B}	5.8 ± 0.1^{a}	39 ± 2^{A}
18:3 ω3	5.4 ± 0.1^{b}	93 ± 3^{C}	6.7 ± 0.0^{c}	46 ± 1^{B}	nd^{a}	nd^{A}
18:4 ω3	6.5 ± 0.1^{b}	112 ± 4^{C}	7.6 ± 0.0^{c}	52 ± 1^{B}	nd^{a}	nd^{A}
20:4 ω3	0.9 ± 0.0^{b}	16 ± 1^{B}	nd^{a}	nd^{A}	nd^{a}	nd^{A}
20:5 ω3	10.2 ± 0.1^{b}	176 ± 4^{C}	5.6 ± 0.0^{a}	39 ± 1^{A}	13.5 ± 0.1^{c}	90 ± 4^{B}
22:6 ω3	nd^{a}	nd^{A}	nd^{a}	nd^{A}	nd^{a}	nd^{A}
Σ PUFA	38.6 ± 0.1^{b}	667 ± 19^{C}	43.9 ± 0.2^{c}	302 ± 6^{B}	20.7 ± 0.2^{a}	138 ± 7^{A}
Σ ω3	23.0 ± 0.2^{c}	396 ± 12^{C}	20.0 ± 0.0^{b}	137 ± 2^{B}	13.5 ± 0.1^{a}	90 ± 4^{A}
Σ ω6	15.7 ± 0.1^{b}	270 ± 7^{C}	23.9 ± 0.2^{c}	164 ± 4^{B}	7.1 ± 0.1^{a}	47 ± 3^{A}
Σ ω3/Σ ω6	1.47 ± 0.01^{b}	1.47 ± 0.01^{B}	0.84 ± 0.01^{a}	0.84 ± 0.01^{A}	1.91 ± 0.03^{c}	1.91 ± 0.03^{C}

The analysis of the relative FA profile (as a percentage of the total FAs) shows important differences between the three seaweed species. Whereas *P. binghamiae* and *H. scoparia*, both brown algae, had a similar weight of saturated FAs (SFA) and PUFA, *O. pinnatifida* was characterized by an overwhelming abundance of SFA. Monounsaturated FAs (MUFA) were not very abundant in any of the studied species. Within PUFA, ω3 PUFA were the most abundant in *O. pinnatifida* and *P. binghamiae*, thus yielding ω3/ω6 ratios clearly higher than one, 1.91 ± 0.03 and 1.47 ± 0.01, respectively. In contrast, *H. scoparia* displayed a very low ω3/ω6 ratio, 0.84 ± 0.01. Regarding the highly unsaturated ω3 PUFA (HUFA), such as EPA and DHA, only EPA was detected and reached high percentages in *O. pinnatifida* and *P. bighamiae*. The former seaweed exhibited the highest content, 13.5 ± 0.1%. This seaweed did not contain less unsaturated ω3 PUFA, such as α-linolenic acid (ALA, 18:3 ω3) and stearidonic acid (SDA, 18:4 ω3). However, these FAs were relevant in the profiles of *P. binghamiae* and *H. scoparia*, being in the 5-8% range. Linoleic acid (18:2 ω6) content differed starkly between species, ranging from less than 2% in *O. pinnatifida* to 13.4 ± 0.1% in *H. scoparia*. Arachidonic acid (20:4 ω6) level also varied from *O. pinnatifida* (5.8 ± 0.1%) to *P. binghamiae* (7.9 ± 0.1%) and *H. scoparia* (9.2 ± 0.2%), but within a narrower range. Furthermore, high contents of palmitic acid (16:0) and substantial contents of myristic (14:0) were determined in all studied seaweed species. However, palmitic acid level in *O. pinnatifida* was approximately double that in *P. binghamiae*, reaching an extremely high percentage, 47.2 ± 0.1%. Contrastingly, oleic acid (18:1 ω9) concentration was highest in *P. binghamiae*, 17.0 ± 0.1%.

The FA concentrations in mg/100 g of the dw seaweed offer an alternative point of view. Firstly, it should be remarked that the total identified FA amount was different between species. While *H. scoparia* and *O. pinnatifida* reached 643-687 mg/100 g dw, *P. binghamiae* total FA amount was much higher, surpassing 1600 mg/100 g dw. The higher abundance of FAs in this latter seaweed entails that the highest absolute concentrations of many FAs were found in *P. binghamiae*, even when their relative percentages were lower.

Indeed, the highest contents of palmitic (427 ± 10mg/100g dw), oleic (293 ± 6 mg/100g dw), linoleic (123 ± 3mg/100g dw), and arachidonic acid (136 ± 4mg/100 g dw) as well as EPA (176 ± 4mg/100g dw) were determined in this specie. Considering human EPA+DHA requirements and considering the possibility of using *P. binghamiae* in food applications, it may be calculated how much freeze-dried seaweed is needed to meet the recommended daily intake of EPA+DHA (500mg/day) according to the American Heart Association (Kris-Etherton et al., 2002). This calculation gives an estimated amount of 284g of sun-dried *P. binghamiae*, which is not high (especially if hydration is considered), but still relevant from a nutritional point of view.

These results are currently submitted to the International Journal of Food Science and Technology for publication (see Annex I).

4.4. Beta-glucan content

The β-glucan (laminarin) content in the selected brown algae (*Petalonia binghamiae* and *Halopteris scoparia*) and red algae (*Osmundea pinnatifida*) is displayed in Table 4.5. This content was low in all studied species, never exceeding 0.2% w/dw. *O. pinnatifida* presented the lowest level (0.02% w/dw) and *P. binghamiae* the highest level (0.18% w/dw).

These results are currently submitted to the International Journal of Food Science and Technology for publication (see Annex I).

Table 4.5. – Total β-glucan content (%, w/dw) of the studied seaweed species *Petalonia binghamiae*, *Halopteris scoparia*, and *Osmundea pinnatifida*. Values are presented as average ± standard deviation. Different lowercase letters within a row correspond to statistical differences (p<0.05) between seaweeds.

Sample	*Petalonia binghamiae*	*Halopteris scoparia*	*Osmundea pinnatifida*
β-glucans (% w/dw)	0.18 ± 0.00^{c}	0.11 ± 0.00^{b}	0.02 ± 0.00^{a}

4.5. Total polyphenol content

The results of *H. scoparia* total phenolic content attained with four different reaction times are displayed in Table 4.6. It is easily observed that after a 15-minute reaction the amount of detected polyphenols is null, and it increases with a higher reaction time up to 1 hour and 30 minutes. After this time there is no visible advantage in maintaining the reaction and may even lead to the degradation of some phenolic compounds.

Table 4.6. – Variation of the phenolic content (mg GAE/100g dw) in *Halopteris scoparia* with the duration of the reaction in the dark. Values are presented as average ± standard deviation.

Halopteris scoparia	15min	45min	1h30min	3h
Absorbance (750nm)	0.06 ± 0.0	0.48 ± 0.0	0.58 ± 0.0	0.57 ± 0.0
Phenolic content (mg GAE/100 g dw)	0.0 ± 0.0	171 ± 0.0	217 ± 0.0	217 ± 0.0

The total polyphenol content of *Petalonia binghamiae*, *Halopteris scoparia*, and *Osmundea pinnatifida* is presented in Table 4.7. The aqueous extract of *H. scoparia* exhibited the highest total polyphenol content, yielding a total of 217 ± 8mg GAE/100g dw. The lowest value was found in an alcoholic extract of *P. binghamiae* (142 ± 3mg GAE/100g dw). With exception of *O. pinnatifida*, it was possible to identify an advantage of aqueous extraction over alcoholic extraction.

These results are currently submitted to the International Journal of Food Science and Technology for publication (see Annex I).

Table 4.7. Total polyphenol content of the studied seaweed species *Petalonia binghamiae*, *Halopteris scoparia*, and *Osmundea pinnatifida*. The values are presented as absolute phenolic content (mg/g dw) and as gallic acid equivalents (mg GAE/100g dw). Values are presented as average ± standard deviation. Different lowercase letters within a row correspond to statistical differences (p<0.05) between seaweeds.

Sample	***Petalonia binghamiae***	***Halopteris scoparia***	***Osmundea pinnatifida***
	Total polyphenol (mg GAE/100g dw)	Total polyphenol (mg GAE/100g dw)	Total polyphenol (mg GAE/100g dw)
Aqueous	174 ± 5^{b}	217 ± 8^{c}	158 ± 5^{a}
Ethanolic	142 ± 3^{a}	169 ± 8^{b}	172 ± 7^{b}

4.6. Antioxidant activity

The antioxidant activity values for aqueous and alcoholic extracts of *Petalonia binghamiae*, *Halopteris scoparia*, and *Osmundea pinnatifida* are shown in Table 4.8. In order to facilitate visual comparison after conversion and statistical analysis, the alternative techniques that were applied to the extracts, DPPH, FRAP, and ABTS, are presented in Figure 4.4. in A, B, and C graphs, respectively. Aqueous extracts generally showed higher activity, especially the aqueous extract of *P. binghamiae*. However, the aqueous extract of *H. scoparia* also exhibited high activity with the ABTS method. Ethanolic extracts showed almost no Ferric Ion Reducing Antioxidant Power.

These results are currently submitted to the International Journal of Food Science and Technology for publication (see Annex I).

Table 4.8. – Antioxidant activity results before conversion. DPPH and ABTS values are shown in percentage of inhibition. FRAP results are not shown in percentage since this method measures de amount of Fe^{III} reduced to Fe^{II} in the reaction, therefore the values are presented in mmol Fe^{II}/L. Values are presented as average ± standard deviation. Different lowercase letters within a row correspond to statistical differences (p<0.05) between seaweeds.

Sample	***Petalonia binghamiae***		***Halopteris scoparia***		***Osmundea pinnatifida***	
	Aqueous extract	Ethanolic extract	Aqueous extract	Ethanolic extract	Aqueous extract	Ethanolic extract
DPPH (% inhibition)	79.3 ± 0.0^{e}	21.8 ± 0.0^{b}	29.4 ± 0.0^{c}	7.9 ± 0.0^{a}	40.6 ± 0.0^{d}	41.8 ± 0.0^{d}
FRAP (mmol Fe^{II}/L)	0.7 ± 0.0^{e}	0.0 ± 0.0^{a}	0.4 ± 0.0^{d}	0.0 ± 0.0^{a}	0.2 ± 0.0^{c}	0.1 ± 0.0^{b}
ABTS (% inhibition)	90.3 ± 0.0^{e}	7.6 ± 0.0^{a}	93.3 ± 0.0^{e}	32.6 ± 0.1^{c}	56.5 ± 0.0^{d}	20.3 ± 0.0^{b}

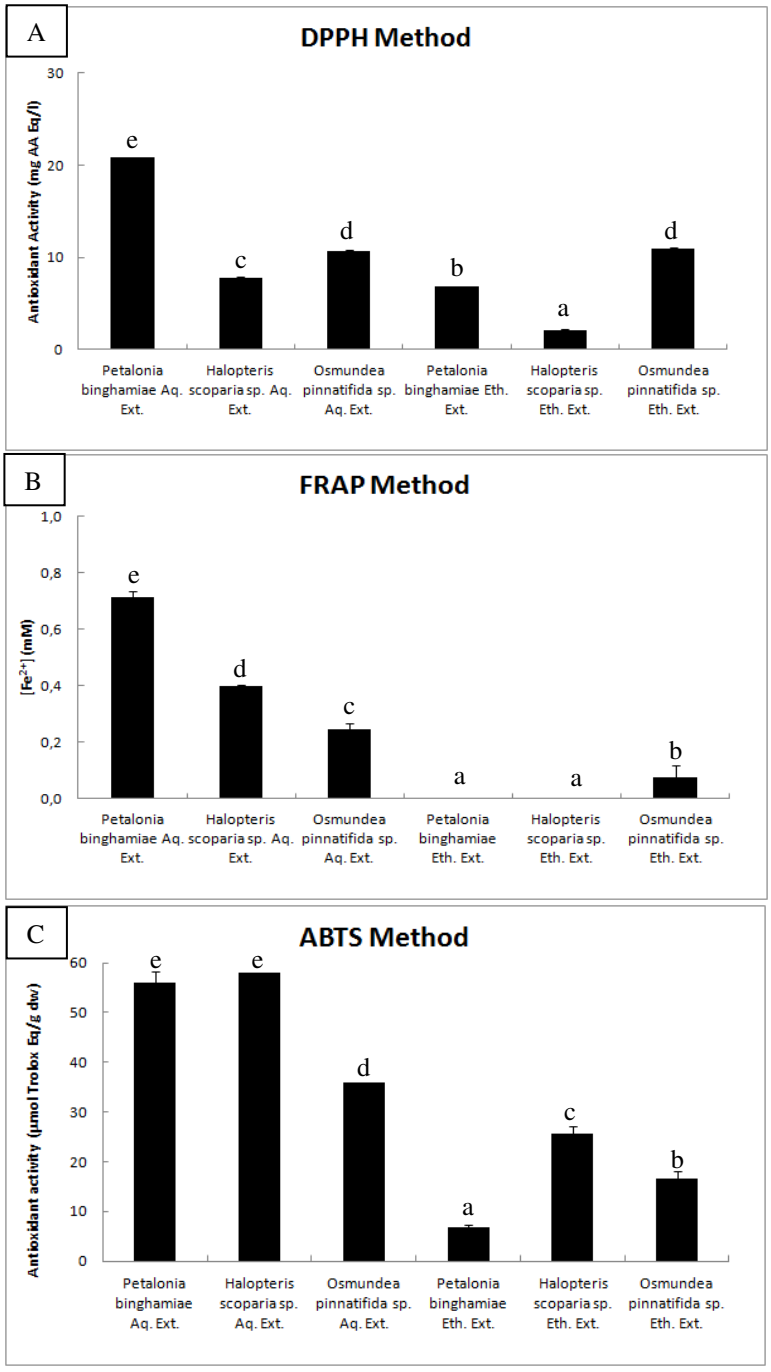

Figure 4.4. - Antioxidant activity as measured by DPPH (mg Ascorbic Acid Equivalent/l) (A), FRAP (mM Fe^{II} eq.) (B), and ABTS (µmol Trolox Equivalent/g dw) (C) methods in aqueous (Aq.) and ethanolic (Eth.) extracts of the studied seaweed species *Petalonia binghamiae*, *Halopteris scoparia*, and *Osmundea pinnatifida*. Different lowercase letters correspond to statistical differences between seaweeds (p<0.05).

4.7. Anti-inflammatory activity

The anti-inflammatory activity of *Petalonia binghamiae*, *Halopteris scoparia*, and *Osmundea pinnatifida*, as measured in percentage of inhibition of the enzyme COX-2, is shown in Table 4.9. Two different extracts were assayed, an aqueous and an alcoholic extract. Extract concentration was 1 mg/ml in DMSO.

Only the alcoholic extract of *H. scoparia* and the aqueous extract of *P. binghamiae* showed inhibitory capacity of cyclooxygenase-2 (COX-2), with values of 79 ± 8% and 40 ± 7%, respectively. These two values were statistically ($p<0.05$) different.

These results are currently submitted to the International Journal of Food Science and Technology for publication (see Annex I).

Table 4.9. – Anti-inflammatory activity (% inhibition of COX-2) in aqueous and ethanolic extracts of the studied seaweed species *Petalonia binghamiae*, *Halopteris scoparia*, and *Osmundea pinnatifida*. Values are presented as average ± standard deviation. nd = not detected. Different lowercase letters within a row correspond to statistical differences ($p<0.05$) between seaweeds.

Extract	***Petalonia binghamiae*** (% inhibition)	***Halopteris scoparia*** (% inhibition)	***Osmundea pinnatifida*** (% inhibition)
Aqueous	40 ± 7^{b}	nd^{a}	nd^{a}
Ethanolic	nd^{a}	79 ± 8^{b}	nd^{a}

5. Discussion

5.1. Proximal composition

The results attained in this study are in most cases similar to those reported in the existing literature for all seaweed species.

The moisture content was low in all macroalgae, which was expected since they were previously sun-dried. The lowest value was recorded for *Halopteris scoparia* (3.2%), which may be due to its own morphology which naturally retains less water than the other two studied species.

The ash content in macroalgae may reach up to 55% (Kumar et al., 2011), and the values obtained were in agreement with this assumption. The values attained for each seaweed were also concordant with other studies for similar species. More specifically, a previous study with *Osmundea pinnatifida* also collected in the Azores islands reports an ash content of 38.5%, being this value practically the same as that obtained in this study (39.4%). Literature on the other two studied species is very scarce, but studies with *Halopteris funicularis* and *Petalonia fascia* obtained ash contents of approximately 43.9% (Lamare and Wing, 2001) and 27.2% (Irkin et al., 2017), respectively, and these results are similar to those obtained in the present study for *Halopteris scoparia* (51.9%) and *Petalonia binghamiae* (27.9%). The ash content of *H. scoparia* stands out from the rest and may be particularly interesting, since it indicates that this macroalgae may be very rich in micro-nutrients. However, this is the only of the three studied species that is considered non-edible, but still may be used in bioremediation or as a bioindicator of quality in marine environments (Patarra et al., 2018).

Regarding protein content, even though this fraction is highly variable on macroalgae, the obtained results seem to be concordant with the pre-existing literature. In brown seaweeds, the protein fraction is usually not higher than 24%, which is verified in both studied species. About red macroalgae, previous studies with *Osmundea pinnatifida* and *Undaria pinnatifida* (Paiva et al., 2014) report protein contents of 20.8% dw and 18% dw, respectively, which are not very far from the value attain for the studied red seaweed (14.4%). However, some studies indicate a much lower content for this same macroalgae (6.8% dw) (Demirel et al., 2012), which might be due to the fact that the specimens used in the current work were collected in October and the ones used in the previous study were collected during winter time.

The total lipid content was also in the expected range of values for macroalgae, usually 1-6% dry weight (Holdt and Kraan, 2011). Kaneniwa (1998) reports a total lipid content of 4.6% for *Petalonia binghamiae*, almost the same value obtained now by the Folch method (4.5%). It is worth mentioning that there are some studies that seem to indicate higher lipid contents for *Halopteris scoparia* and *Osmundea pinnatifida*. Paiva et al. (2014) obtained a crude fat content of 7.5% for Azorean *Osmundea pinnatifida*, but it should be noted that the extraction method used (Soxhlet) was different from that used in the current study and that the samples were harvested in January, being the winter a period when seaweeds might accumulate reserve lipids.

Finally, regarding carbohydrate content, there is very little literature due to the difficulty of extracting and accurately quantifying these compounds from algal cell walls. It is known that marine macroalgae are very rich in polysaccharides, being their total percentage up to 76% dry weight in some species (Holdt and Kraan, 2011). The values obtained were high for all species, especially in the case of *Petalonia binghamiae* (48.3%), representing almost half of the total seaweed's dry weight. A study on *Petalonia fascia* indicated a carbohydrate content of 39.9%, which shows some level of concordance inside

the genus *Petalonia* (Demirel et al., 2012). Paiva et al. (2014) attained a total carbohydrate content of 17.6% for *Osmundea pinnatifida*, being this significantly lower than what was estimated in this study (37.9%), but since both values were obtained by difference, this might be in part due to seasonal variations in the other parameters of the algal proximate composition.

5.2. Lipid classes

It has been recognized that lipid composition of algae is variable throughout their life cycle and is affected by environmental factors, such as temperature, light and nutrient availability. For example, in *Undaria pinnatifida* the total lipid content is higher during winter and autumn as compared to summer, and high triacylglycerol contents are observed in spring and summer, while polar lipids (PL and GL) depended on the algal development stages throughout the year (Kumari et al, 2013).

Most studies in fresh macroalgae indicate that polar lipids (PL) are the predominant lipid class, with values going up to 94% of total lipids in some species (Nelson et al., 2002). However, this was not verified for the studied sun-dried biomass. The polar lipid content attained for the three studied seaweed species ranged from 8.7% in *P. binghamiae* to 21.1% in *O. pinnatifida*. This is probably due to the sun exposure treatment that was applied to the samples after harvest, which is not ideal for preserving these lipid classes. It is also possible that the activity of lipases, such as glycolipase and phospholipase, is high in these macroalgae, causing the degradation of the polar lipids to free fatty acids. This is in agreement with the high FFA levels verified for all studied species. To prevent this issue, some authors defend that treatments, such as enzyme denaturation, should be carried out before lipid extraction (Saito et al., 2010). Freezing the samples immediately after being harvested may also be a better way to preserve lipid classes. The glycolipid standard available for use in this assay (PSY) is also the not ideal one, since the predominant glycolipids in macroalgae are MGDG and DGDG (Dembitsky et al., 1991). Nevertheless, there are studies in green macroalgae (*Rhizoclonium riparium*, *Ulva intestinalis*, and *Chaetomorpha linum*) that report non-polar lipid levels higher than 70% of the total lipid content, as obtained in the present study (Cardoso et al., 2017).

P. binghamiae exhibited the highest TAG content, which is concordant with the fact that this species is the one with highest total lipid content (4.5%) and seaweeds usually accumulate lipids in this form. Regarding diacylglycerols, very low amounts of DAG-1,2 were quantified (around 2% for all species), and it was not possible to clearly identify the DAG-1,3 class in any sample. This may be due to the fact that this lipid class is probably very scarce in the samples. DAG 1,2 form more easily from a chemical point of view than DAG-1,3, more specifically, at least in a 2:1 ratio. Therefore, the DAG-1,3 class should correspond to only around 1% or less of the total classes quantified for these macroalgae, which makes it difficult to visualize on the TLC plate. The fact that there is an unidentified band of considerable size near the zone where the DAG-1,3 should be eluted may also make it impossible to identify and quantify this class.

Finally, sterol content in seaweeds usually ranges from around 3 to 8% of total lipids and tends to decrease during autumn in most species (Nelson et al., 2002). This is true for all studied species, since after considering the results attained with the two elution mixtures the sterol content varied from 2.6% in *P. binghamiae* to 5.9% in *O. pinnatifida*. Even though a cholesterol standard (CH) was used for having similar structure and polarity, it is important to acknowledge that the predominant sterol in brown and red macroalgae is fucosterol.

5.3. Fatty acid profile

Petalonia binghamiae, Halopteris scoparia and *Osmundea pinnatifida* do not belong to the group of less than 20 seaweed species that represent 90 % of the algal biomass subjected to commercial exploitation (Pereira, 2016), thus their fatty acid profile has not been the focus of many studies. The low levels of absolute FA contents agree with the existent seaweed literature, both for brown macroalgae of the *Ectocarpales* order (*P. binghamiae*) or of the *Sphacelariales* order (*H. scoparia*) and for red macroalgae of the *Ceramiales* order (*O. pinnatifida*) (Gosch et al., 2012). Most brown seaweed species have low ω3/ω6 ratios, uasually lower than one, and low EPA and DHA contents (Gosch et al., 2012; Paiva et al., 2014; Rodrigues et al., 2015). Red seaweed typically display a lower lipid level than brown seaweed (Gosch et al., 2012) and, sometimes, relatively high ω3/ω6 ratios, due to levels of EPA ranging from moderate to high percentages of total FAs (Rodrigues et al., 2015). An outstanding example is the red macroalgae *Grateloupia turuturu* with almost 30% EPA (Rodrigues et al., 2015).

Concerning *P. binghamiae*, a previous work on *P. fascia* reported a very similar relative FA profile (Khotimchenko, 1998). This is remarkable, since only EPA content differed, being higher in *P. fascia*, 15.4%. The palmitic (23.5%), oleic (17.7%), linoleic (5.5%), arachidonic (8.0%), α-linolenic (5.3%), and stearidonic acid contents (8.6%) in *P. fascia* were very similar to the ones obtained in this current study for *P. binghamiae* (Khotimchenko, 1998). This might indicate a high degree of conservation of the FA profile across species and geographical areas (Azores, Atlantic Ocean *vs* Russian Far East, Pacific Ocean) in the case of the genus *Petalonia*. However, another study on *P. fascia* (Akgül et al., 2015) showed large differences from *P. binghamiae*. These differences may be caused by the high variability existent in seaweeds during their life cycle or due to the environmental conditions.

Regarding *H. scoparia*, a study on *H. filicina* found a very unbalanced FA profile, with a very high amount of SFA, since palmitic acid and stearic acid (18:0) contents were approximately 43% and 21% of total FAs, respectively (Silva et al., 2013). This diverges strongly of the much more balanced FA profile determined for *H. scoparia*. Absolute FA concentrations were also quite different. A study encompassing *H. scoparia* showed results more similar to those of the current work (Pereira et al., 2012), where the total FA amount was approximately 550mg/100g dw and overall SFA:MUFA:PUFA was 35:14:51, not very different when compared to the Azorean *H. scoparia*. Nonetheless, there were differences, since linoleic acid and EPA contents were higher and ω3/ω6 ratio lower in the Algarve *H. scoparia* (Pereira et al., 2012) than in the Azorean *H. scoparia*.

Finally, regarding *O. pinnatifida*, there is a study on this species (Paiva et al., 2014) that found out a FA profile with an abundance of palmitic acid (46% of total FAs), SFA (57%), oleic acid (14%), and MUFA (21%) relatively similar to the profile obtained for this same seaweed in the current study. However, instead of EPA, this study (Paiva et al., 2014) reported approximately 16% of 20:3 ω3. This is contradicted by another work on *O. pinnatifida* from the mainland Portuguese coast (Rodrigues et al., 2015), which determined 15.6 ± 0.1% (of total FAs) for EPA. Furthermore, 14:0 (approximately 7%), palmitic (49%), oleic (13%), linoleic (1%), and arachidonic acid levels (5%) as well as total SFA (58%), MUFA (19%), PUFA (23%), ω3 PUFA (16%), and ω6 PUFA contents (7%) were remarkably close to the Azorean *O. pinnatifida*. The scarcity of information about this macroalgae species does not enable to conclude if there were methodological problems in any study or seasonal/geographic factors accounted for the differences.

The FA profile and total FA amounts of seaweeds vary significantly throughout the year (Manivannan et al., 2008). Besides sampling period, location may account for any differences (Munier et al., 2013; Rodrigues et al., 2015). Nevertheless, the current study's results and the pre-existent literature seem to support some general trends observed in specific taxa, for instance, that brown seaweed species may be rich in myristic (14:0), palmitic, oleic, and linoleic FAs (Heiba et al., 1997; Akgül et al., 2015).

5.4. Beta-glucan content

The values obtain in this study for the β-glucan content are much lower than those found in certain brown seaweed species, such as *Laminaria hyperborea* or *Ascophyllum nodosum*, whose content may be higher than 6% w/dw (Kadam et al., 2015). Red seaweed species do not accumulate this carbohydrate (Kraan, 2012), thus matching the attained results for *O. pinnatifida*. All in all, the studied macroalgae species are not a good source of laminarin. In addition, it should be noted that this carbohydrate content varies with factors such as harvesting season and geographical location (Kadam et al., 2015). Laminarin is absent during the period of fast growth in Spring, but, in Autumn and Winter, it may represent up to 35% of the dried weight of the fronds (Rinaudo, 2007). In this regard, it is worth mentioning that the main reserve polysaccharides of brown macroalgae are laminarin and mannitol (Kolb et al., 1999).

5.5. Total polyphenol content

The total polyphenol content measured for all three studied seaweed species overlap with the range reported in the relevant literature for biomass rich in polyphenols, 100-500mg GAE/100g dw (Farasat et al., 2013). Values are also equivalent to those reported for *P. binghamiae* from Japan (Kuda et al., 2006) and *H. scoparia* from Algeria (Fellah et al., 2017). However, while this Algerian *H. scoparia* had a polyphenol content of 105 ± 1mg GAE/100 g dw (half the Azorean *H. scoparia* content) in the Autumn, Summer values were much lower, below 50mg GAE/100g dw (Fellah et al., 2017). The Azorean *H. scoparia* was also harvested in the Autumn (see 3.1). With exception of ABTS (particularly in alcoholic extracts), there was no correlation between polyphenol content and the antioxidant activity (see below).

Comparison to literature shows how meaningful the differences in polyphenol content are, even for the same seaweed species. Such divergence may be connected to the variation of UV radiation level with geographic location and season, since higher phenolic contents in macroalgae are associated with higher UV exposure (Bischof et al. 2006). However, this was not found in the seasonal study of the Algerian *H. scoparia* (Fellah et al., 2017). Though it is assumed that seaweeds synthesize more phenolic substances to scavenge the reactive oxygen species produced by UV radiation, other factors may be influential. Indeed, another possible factor influencing phenolic content - and thus antioxidant activity - may be the FA composition of the lipid fraction of the algae specimens. Higher PUFA content (in % of total FAs) in *H. scoparia* (Table 1) can be matched to a higher phenolic content. It is known that PUFA are more prone to oxidation than SFA and monounsaturated FAs (MUFA) (Tao, 2015), thereby generating radicals and other substances with a potentially harmful effect on the cells.

Therefore, living organisms must defend themselves against such compounds, being polyphenols a possible response, given their antioxidant properties. Regarding this, it should be noted that *P. binghamiae* had the highest absolute content of PUFA, which would be a reason for containing the highest total polyphenol content. Nonetheless, it can be hypothesized that a higher oxidative susceptibility is mainly derived from the relative weight of PUFA in the whole lipid fraction.

5.6. Antioxidant activity

Regarding the DPPH assay, the highest values were measured for the aqueous extract of *P. binghamiae*, with a DPPH inhibition of 79 %, which corresponds to 20.8 ± 0.1mg AA Eq/l. For both brown seaweed species, antioxidant activity was higher for the aqueous extracts than the alcoholic extracts. These results had a considerable overlap with FRAP values, since the highest FRAP inhibition was determined for the aqueous extract of *P. binghamiae* and aqueous extracts were also stronger antioxidants than the alcoholic extracts. The alcoholic extracts attained from *P. binghamiae* and *H. scoparia* did not even display any antioxidant power. ABTS values trends had similarities with the DPPH and FRAP ones. More specifically, aqueous extracts had a stronger antioxidant activity than the alcoholic extracts and the highest antioxidant activities were found in aqueous extracts of *P. binghamiae* with 56.0 ± 2.4µmol Trolox Eq/g dw and *H. scoparia* with 58.1 ± 0.1µmol Trolox Eq/g dw. Regarding alcoholic extracts, *H. scoparia* ABTS values exceeded those of the other two studied species.

The attained values show that there is substantial antioxidant activity in extracts of these seaweed species. There is concordance with the relevant literature, for instance, Japanese *P. binghamiae* aqueous extracts had much higher DPPH levels than alcoholic extracts and this was paralleled by total phenolic content (Kuda et al., 2006). Furthermore, methanolic extracts of *O. pinnatifida* had a similar antioxidant activity as measured by DPPH (Paiva et al., 2014). Another study on a red macroalgae species, *Kappaphycus alvarezii* variant Green Flower (Ling et al., 2013), reported a DPPH radical scavenging activity of 30% for a methanolic extract of 6%, w/v, which compares to a DPPH value of 41-42 % (equivalent to 10.7-11.0 mg AA Eq/l) for seaweed extracts of 5%, w/v, in Azorean *O. pinnatifida*. For ABTS, values similar to those of the aqueous extracts of studied brown seaweeds were found in the methanolic extract of the green algae species, *Acetabularia acetabulum*, approximately 50 µmol Trolox Eq/g dw (Sivaramakrishnan et al., 2017).

There was a considerable degree of convergence in the antioxidant activity, as determined by different methodologies. However, a divergence that can be mentioned would be the relative strengthening of the ABTS measured antioxidant activity of *H. scoparia* when compared to the DPPH methodology. Anyway, given the fact that these methodologies are not equivalent and reflect different antioxidant properties, the convergence is quite relevant. Indeed, whereas FRAP only responds to antioxidants that operate through single electron transfer, ABTS detects antioxidant activity by single electron transfer (direct reduction of $ABTS^{+\bullet}$) or radical quenching by hydrogen atom transfer (Prior et al. 2005). Moreover, ABTS has been deemed as more sensitive than DPPH, usually yielding different results (Martysiak-Żurowska and Wenta, 2012). Accordingly, antioxidant observations in the current work are thus mutually reinforced and, as such, may be deemed as more reliable.

Only ABTS values had some degree of correlation with total polyphenol content (high levels for *H. scoparia* and higher values for aqueous extracts than alcoholic extracts). Since polyphenols act as antioxidants through single electron transfer and through hydrogen atom transfer, being their bond dissociation energy and ionization potential the two basic physicochemical parameters that enable to assess the potential efficacy of each process, respectively (Makris and Boskou, 2014; Quideau et al., 2011), ABTS may be more sensitive to the presence of these compounds in the seaweed biomass.

5.7. Anti-inflammatory activity

The number of studies on the anti-inflammatory activity of seaweeds is low and the assay techniques are very variable, involving *in vitro* tests and *in vivo* models (Jin et al., 2006; Margret et al., 2009; Yang et al. 2010; Bitencourt et al., 2015; McCauley et al., 2015). This raises difficulties in the comparison across studies. Nevertheless, there are studies pointing to the existence of anti-inflammatory activity in brown seaweed species (Yang et al. 2010). More specifically, this latter study reported that *P. binghamiae* reduced the lipopolysaccharide-induced expressions of inducible nitric oxide synthase and COX-2 at the protein level in a concentration-dependent manner, as determined by Western blotting. The latter effect is different from that measured in the current study, where the activity of the enzyme was reduced by inhibition. Some other brown macroalgae species, such as *Undaria pinnatifida*, have been claimed to promote anti-inflammatory activity (Hwang et al., 2014). This was observed in an alcoholic extract. An identical methodology based on inhibition of COX-2 was used for green seaweeds (Ripol et al., 2018) and there was anti-inflammatory activity in 10%, w/v aqueous extracts, whose residue was dissolved in DMSO in a concentration of 1mg/ml. The inhibition of COX-2 varied between 31 and 45%, which is similar to *P. binghamiae* aqueous extract in current study.

Different classes of substances may cause the anti-inflammatory effects, such as phenolic compounds, carotenoids, phytosterols, alkaloids or polysaccharides, as exemplified by a sulfated polysaccharide from *Caulerpa cupressoides* (Rodrigues et al., 2012). Though ω3 PUFA and, particularly, EPA have anti-inflammatory effects (Calder, 2010; Tanaka et al., 2014), their action requires *in vivo* systems, which was not the case in the COX-2 inhibition assay used in the current study. Moreover, there is no correlation between total polyphenol content (Table 2) in the extracts and their anti-inflammatory activity. Therefore, the anti-inflammatory activity of *P. binghamiae* and *H. scoparia* may be the result of different substances, more soluble in ethanol in the latter seaweed and more soluble in water in the former seaweed. In the case of *H. scoparia*, this seems to exclude polysaccharides, given their low solubility in ethanol. Other compounds, such as carotenoids and phytosterols, known to be soluble in alcohols, may be the anti-inflammatory bioactives in the case of *H. scoparia*.

6. Conclusion

Several studies have already shown the biotechnological potential of seaweeds in general. The great intrinsic variety found in macroalgae, both intra and interspecific, as well as its own structure and morphology and sometimes the lack of appropriate literature are major challenges in this area of study. The applied techniques have to be constantly adapted and there is a great need to look for methodologies that are as universal as possible for the study of these organisms. However, this work offers a more detailed perspective on three seaweed species (*Petalonia binghamiae*, *Halopteris scoparia*, and *Osmundea pinnatifida*) with commercial importance in Portugal.

The three seaweed species had a proximate composition concordant with the pre-existing literature for similar species. *Halopteris scoparia* showed an especially high mineral content, thus indicating possible future applications in bioremediation or heavy metal control in marine environments. All species had a low fat content as is expected for macroalgae, but *Petalonia binghamiae* stood out with a total lipid content of 4.5% dw. Their contribution as an energy source appears to be low but some lipid fractions might have important activities and are worth studying. The edible seaweed *P. binghamiae* also had a very high carbohydrate content. This is an interesting result because most polysaccharides in macroalgae are dietary fibers and even though these are not digested by the human body they have a positive impact on the digestion process which means this seaweed might have important pre-biotic activity.

Contrary to what is described in most studies for fresh samples, all macroalgae exhibited a higher percentage of non-polar lipids, possibly due to the degradation of phospholipids and glycolipids by the sun treatment, causing an increase of free fatty acids (FFA). All species revealed a high TAG content, which might be interesting from the perspective of using these macroalgae's fatty acid esters for biodiesel production, produced by transesterification of triacylglycerols. The DAG-1,2 content was low in all samples. Sterol content was significant specially in *O. pinnatifida* (5.9%), meaning that it is worth studying the potential use of this seaweed's fucosterol in various biological therapeutics, due to its wide range of bioactivities, such as antidiabetic, antioxidant and antihistaminic. It can also be concluded that TLC is not a very precise method, and it would be important to repeat this analysis using high performance thin layer chromatography (HPTLC). Future analyzes should also include the quantification of monoacylglycerols (MAG), which were not quantified in this study.

The fatty acid (FA) profile was characterized by abundance of saturated FA (SFA) in the case of *O. pinnatifida* and a similar weight of SFA and polyunsaturated FA (PUFA) in the other two species. Monounsaturated FA' (MUFA) share was relatively small in all species. Within PUFA, ω3 PUFA were the most abundant in *O. pinnatifida* and *P. binghamiae*, thus yielding ω3/ω6 ratios clearly higher than one. The palmitic acid (16:0) content was very high and the myristic acid (14:0) content was substantial in all species. Oleic acid (18:1 ω9) level was also high, particularly in *P. binghamiae*, reaching 16.95 ± 0.05% of total FAs. Linoleic acid (18:2 ω6) content was in the 7-13% range with exception of *O. pinnatifida*, whose linoleic acid content was low, 1.34 ± 0.01%. Arachidonic acid (20:4 ω6) level was similar or higher than linoleic acid level in all studied species. Regarding ω3 PUFA, eicosapentaenoic acid (EPA, 20:5 ω3) was the most important (10-14%) with exception of *H. scoparia*, which was richer in stearidonic acid (SDA, 18:4 ω3) and α-linolenic acid (ALA, 18:3 ω3) than in EPA, 7.60 ± 0.03% and 6.73 ± 0.03% vs 5.63 ± 0.03%, respectively. The highest EPA concentration was found in *P. binghamiae* (176 ± 4 mg/100 g dw). These are promising results, since some of these fatty acids can cause positive physiological changes in the human body. For example, EPA is essential as a structural component of cell membranes and also has a significative role in lowering blood lipids, as well as mediating biochemical and physiological responses. Additionally,

it is important to notice that the major sources of EPA and other important ω3 fatty acids in human diets are marine products.

Concerning other bioactive compounds, while β-glucans (laminarin) were only detected at trace levels, polyphenols were present at non-negligible levels, reaching 140-220mg/100 g dw, reinforcing the idea that these macroalgae have antioxidant capacity, which was also tested. Aqueous extraction seems to be more effective for these compounds.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Ion Reducing Antioxidant Power (FRAP), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) methods showed some antioxidant activity, especially in aqueous extracts of *H. scoparia* and *P. binghamiae* assessed by ABTS. Ethanolic extracts of *H. scoparia* and aqueous extracts of *P. binghamiae* showed inhibitory capacity of cyclooxygenase-2 (COX-2), between 40 and 79%, thus indicating some promising anti-inflammatory activity. This study is a first step towards the possible future application of these algae in nutraceutical or cosmetic products.

To conclude, it is important to acknowledge that sun-drying, is not the ideal processing when it comes to compounds such as phospholipids and glycolipids, but even in these conditions the algal products are left with substantial nutritional value of protein, minerals, ω3 and ω6 fatty acid contents, and considerable levels of antioxidant and anti-inflammatory activities. There is a lot of potential worth exploring in the studied Azorean macroalgae that may lead to many different applications in the near future.

7. Future perspectives

Although this work gave an important insight on composition and bioactivity of *Petalonia binghamiae, Halopteris scoparia* and *Osmundea pinnatifida*, there are still a lot of questions that require further study.

First of all, due to the difficulty in breaking the cellular structure of macroalgae, it would be important to test different and maybe more efficient extraction methods, for instance ultrasounds or ionic fluids. This is of particular importance when it comes to the quantification of polysaccharides, such as laminarin or dietary fiber.

A detailed mineral profiling would also be crucial, both for edible and non-edible seaweeds. Regarding edible macroalgae, it is essential to assess not only the nutraceutical potentialities that may exist in its mineral content, but also if there are no hazards associated with toxic metals that can be accumulated.

The quantification of other compounds with possible bioactivities, such as fucoxanthin, fucans and phycobiliproteins, is also of major interest. In the same way, some other activities, like anti-diabetic or cytotoxic, should be tested in all studied species.

All the analysis implemented should also be performed on fresh (not sun-dried) samples, in order to obtain a comparative profile of the two conditions. This is of particular importance for the study of the lipid classes, since phospholipids and glycolipids were not possible to quantify in the sun-dried samples.

On another level, since vegetable matter is not always fully digested and absorbed by the human organism, a study of bioacessibility *in vitro* and bioavailability *in vivo* may help to give a more real perspective on how the different algal components are absorbed in digestion.

The attractive properties of macroalgae are creating an expanding market for these products and some other new challenges arise. Seaweed producers are now facing the problematic of harvesting or growing algae on a large scale without harming any further the marine environment. Scientific studies regarding the ecological impact of such activities are therefore essential to assess the real impact of such activities for the ecosystems.

Finally, all seaweed species and their bioactive molecules potentially used in pharmaceutical, nutraceutical or food industries must meet certain consumer safety regulations. In some European countries there are defined lists of seaweed species authorized for human consumption and rigorous maximum allowed levels of toxic metals have been defined for all edible macroalgae. But in others, such as Portugal and Spain, there is no specific legislation regarding algae products. With the growing popularity of functional foods and the fast advances in the pharmaceutical industry, it is crucial to review the legislation and monitor the development of these new products, not to prevent their entrance in the market but to ensure that it is made in a totally safe way.

8. References

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9. Annexes

Annex I.

Azorean Macroalgae (*Petalonia binghamiae*, *Halopteris scoparia*, and *Osmundea pinnatifida*) Bioprospection: A Study of Lipid Profiling and Bioactivity

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Abstract

The Azores archipelago (in mid-Atlantic) has a substantial variety of seaweed species. In particular, the macroalgae species *Petalonia binghamiae, Halopteris scoparia*, and *Osmundea pinnatifida* are still undervalued and not thoroughly studied. As a contribution to the bioprospection of these three species, lipid composition and relevant bioactivities were determined. The three seaweed species had a low fat content and their fatty acid (FA) profile was characterized by abundance of saturated FA (SFA) in the case of *O. pinnatifida* and a similar weight of SFA and polyunsaturated FA (PUFA) in the other two species. Monounsaturated FA' (MUFA) share was relatively small in all species. Within PUFA, ω3 PUFA were the most abundant in *O. pinnatifida* and *P. bighamiae*, thus yielding ω3/ω6 ratios clearly higher than one. The palmitic acid (16:0) content was very high and the myristic acid (14:0) content was substantial in all species. Oleic acid (18:1 ω9) level was also high, particularly so, in *P. binghamiae*, reaching 16.95 ± 0.05 % of total FAs. Linoleic acid (18:2 ω6) content was in the 7-13 % range with exception of *O. pinnatifida*, whose linoleic acid content was low, 1.34 ± 0.01 %. Arachidonic acid (20:4 ω6) level was similar or higher than linoleic acid level in all studied species. Regarding ω3 PUFA, eicosapentaenoic acid (EPA, 20:5 ω3) was the most important (10-14 %) with exception of *H. scoparia*, which was richer in stearidonic acid (SDA, 18:4 ω3) and α-linolenic acid (ALA, 18:3 ω3) than in EPA, 7.60 ± 0.03 % and 6.73 ± 0.03 % *vs* 5.63 ± 0.03 %, respectively. The highest EPA concentration was found in *P. binghamiae* (176 ± 4 mg/100 g dw). Concerning other bioactives, while β-glucans (laminarin) were only detected at trace levels, polyphenols were present at non-negligible levels, reaching 140-220 mg/100 g dw. The 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Ion Reducing Antioxidant Power (FRAP), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) methods showed some antioxidant activity, especially in aqueous extracts of *H. scoparia* and *P. binghamiae* assessed by ABTS. Ethanolic extracts of *H. scoparia* and aqueous extracts of *P. binghamiae* showed inhibitory capacity of cyclooxygenase-2 (COX-2), between 40 and 79 %, thus indicating some anti-inflammatory activity.

Keywords: *Petalonia binghamiae*; *Halopteris scoparia*; *Osmundea pinnatifida*; lipid composition; antioxidant activity; anti-inflammatory activity;

Figure 9.1. First page of the document submitted to the Journal of Food Science and Technology for publication.

Annex II.

Total lipid extraction: Method comparison

The results of total lipid extraction by the two tested methods are shown in Table 9.1. There was a notorious difference between the two methods, being the values attained by the Bligh & Dyer method significantly lower (around half) than those obtained by the Folch method.

The values obtained by the Folch method are closer to those portrayed in the existing bibliography on these species. For example, Kaneniwa (1998) obtained a total lipid content of 4.6% for *Petalonia binghamiae*, almost the same value obtained now with the Folch method (4.5%). Some studies on the other two macroalgae also indicate that their total lipid content is higher than the attained with the Bligh & Dyer assay (Orhan et al., 2003; Paiva et al., 2014). For this reason, Folch was chosen as the lipid extraction method for the remaining analyses and the values obtained by this assay were those used in the final proximal composition of the three macroalgae species.

Table 9.1. – Total lipid content as extracted by two different methods: Bligh & Dyer and Folch. Values are presented as average ± standard deviation.

	Petalonia binghamiae	***Halopteris scoparia***	***Osmundea pinnatifida***
Bligh & Dyer (g/100g dw)	2.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
Folch (g/100g dw)	4.5 ± 0.3	1.9 ± 0.1	1.6 ± 0.1

Annex III.

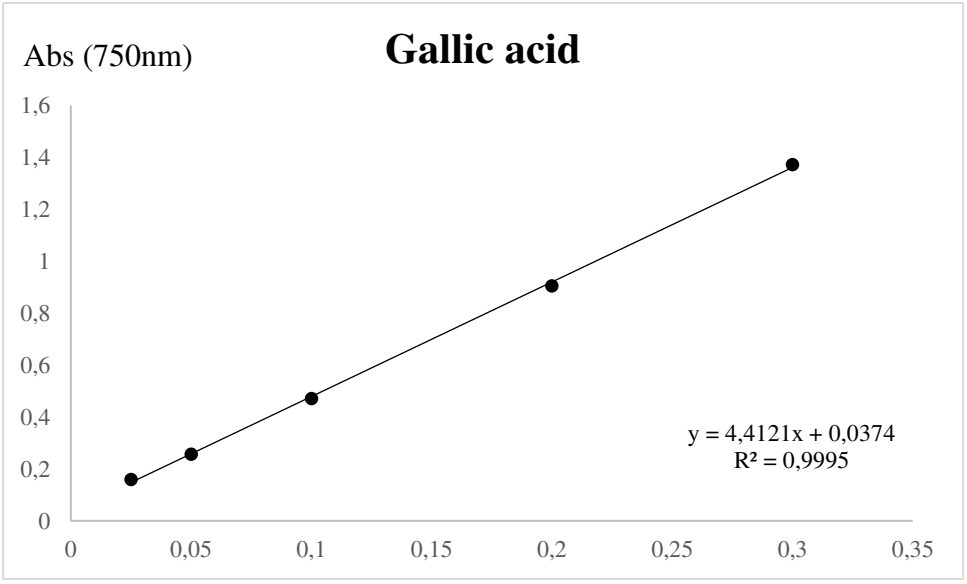

Figure 9.2. Gallic acid calibration curve for polyphenol quantification.

Annex IV.

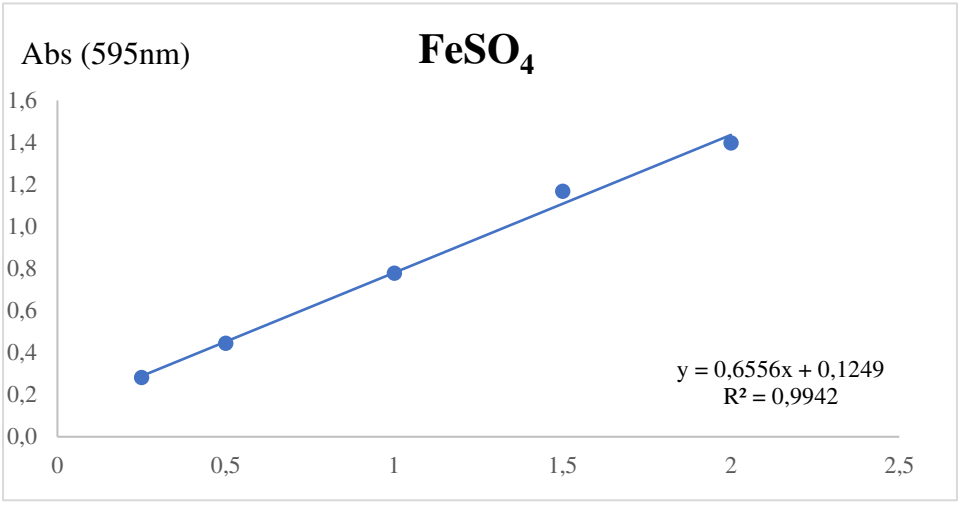

Figure 9.3. Iron (II) sulphate calibration curve for the FRAP assay.